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# PHYSIOLOGICAL REVIEWS

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PHYSIOLOGICAL REVIEWS

VOL. 25 OCTOBER, 1945 No. 4

# CONTROL OF THE SECRETION OF ANTIDIURETIC HORMONE FROM THE PARS NERVOSA OF THE PITUITARY GLAND

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Since the publication of the observations of Frank (52), v. den Velden (186), v. Konschegg and Schuster (103) and Motzfeldt (139) it has many times been confirmed that extracts of the posterior lobe of the pituitary gland contain an antidiuretic rather than a diuretic substance as had originally been thought (116). This review is written from the standpoint that the antidiuretic substance is a hormone (ADH) formed in the pars nervosa of the pituitary gland, that it is liberated into the circulation, and controls the rate of loss of water by a specific action on the kidneys. That this is so is proved by many facts, of which the chief are briefly as follows. A potent antidiuretic substance can be extracted from all normal mammalian posterior lobes (59); glands in which the pars nervosa is atrophied, but the pars intermedia is normal, are practically inactive (50) (51); diabetes insipidus can occur in the presence of a normal pars intermedia (51); the antidiuretic substance is effective in the minute quantities typical of hormones (75) (146) (180); the antidiuretic action persists after denervation or isolation of the kidneys and is effective in the great majority of cases of diabetes insipidus. This is not the place for further discussion on this aspect of the subject. References to the literature may be found in Van Dyke (184), Fisher, Ingram and Ranson (51) and in volumes XVII and XX of the Research Publications of the Association for Research in Nervous and Mental Disease (151) (89). This review is concerned with those observations and experiments which help us to understand how the liberation of the ADH from the gland is regulated.

When referring to that part of the pituitary gland developed from the nervous system the term pars nervosa will be used. The term posterior lobe will be used where the pars nervosa together with the pars intermedia is meant.

As yet we have but scant knowledge of the means by which the chain of events is initiated which leads to an alteration in the rate of excretion of water by the kidneys. Possibly the factor involved is a change in concentration of water in the blood and tissues in relation to one or more dissolved substances. In support of this possibility are the findings of Priestley (153), (154), Margaria (121), Smirk (171), Heller and Smirk (83) and Baldes and Smirk (3) that after the ingestion of water measurable changes occur in the electrical conductivity and water concentration of the blood. That the composition of the blood rather than of the tissues is important as a stimulus seems probable from the work of

Baird and Haldane (2) who found that water diuresis could be induced at a time when the tissues contained an excess of salt. Baldes and Smirk (3) observed what was apparently an adaptation to changes in the water concentration of the blood. They found that if the osmotic pressure of the blood was raised slowly, ingested water resulted in a diuresis although the total osmotic pressure was not below normal limits, and conversely that a slow decrease in the osmotic pressure of the blood did not necessarily lead to a diuresis. There seemed to be a relation between the rate of application of the stimulus and the response.

By what structures in the pars nervosa the ADH is produced is uncertain, whether from the specialised neuroglia cells, the pituicytes (18), or at the nerve terminations (155). That the ADH is made in the pars nervosa and not in the neighbouring pars intermedia is probable since no antidiuretic substance can be extracted from a posterior lobe in which the pars nervosa is atrophied when the pars intermedia is normal (50) (73) (74).

The next question to be answered is whether the pars nervosa is regulated by some local change in its environment, such as the composition of the blood flowing through it, or indirectly by the nervous system and a distant receptor organ. The site of the receptors which respond to changes in the water content of the blood is still undetermined, but many workers since C. and M. Oehme (144) have shown that neither the renal nor the splanchnic nerves subserve this function (101) (163) (194). Janssen (94) showed that the spinal nerves below the lower cervical region were not involved, and both he and Motzfeldt (139) excluded any participation of the vagus below the mid-cervical region. The supraoptic and paraventricular nuclei, which, as will be described later, supply most of the nerve fibres going to the pars nervosa, have an unusually rich capillary supply in particularly close contact with the nerve cells. It has tentatively been suggested that these nerve cells may be stimulated by alterations in the composition of the blood and that they may respond by bringing about changes in pars nervosa activity (49). This is only a suggestion and is not supported by any experimental evidence. Vasquez-Lopez (185), on the other hand, believes the gland itself to be a sensory organ and bases this opinion on the arrangement and large number of nerve fibres in relation to the number of cells, the appearance of the cells and the vascular pattern. He suggests no reason for the presence in the posterior lobe of a potent antidiuretic substance. If indeed the pars nervosa be merely a sensory organ then the interpretation of many well-proved facts becomes exceedingly difficult. If, on the other hand, the pars nervosa be an endocrine gland under the control of the nervous system then many anatomic, experimental and clinical observations may easily be correlated. Anatomically, Cajal's (23) observation that the pars nervosa has an ample nerve supply arising from hypothalamic cells (67) (68) has frequently been confirmed. Experimentally, injury to certain parts of the central nervous system brings about a condition in every way similar to diabetes insipidus more effectively than does injury localised to the pars nervosa itself (24); Marx (123) demonstrated that water diuresis may occur as the result of suggestion during hypnosis; exercise brings about an inhibition of diuresis due to its emotional element (164); certain

sensory stimuli can inhibit water diuresis (179); the rate of excretion of water is markedly affected by the exhibition of a number of narcotics (53). Clinically, Leschke's review (107) presents a good case for the involvement of the central nervous system in cases of diabetes insipidus.

ANATOMY. First the anatomical findings on the innervation of the posterior lobe will be considered. The general conclusions to be drawn are as follows. The pars nervosa is richly innervated by fibres from the supra-optic, paraventricular and certain other hypothalamic nuclei. The supra-optic nuclei and

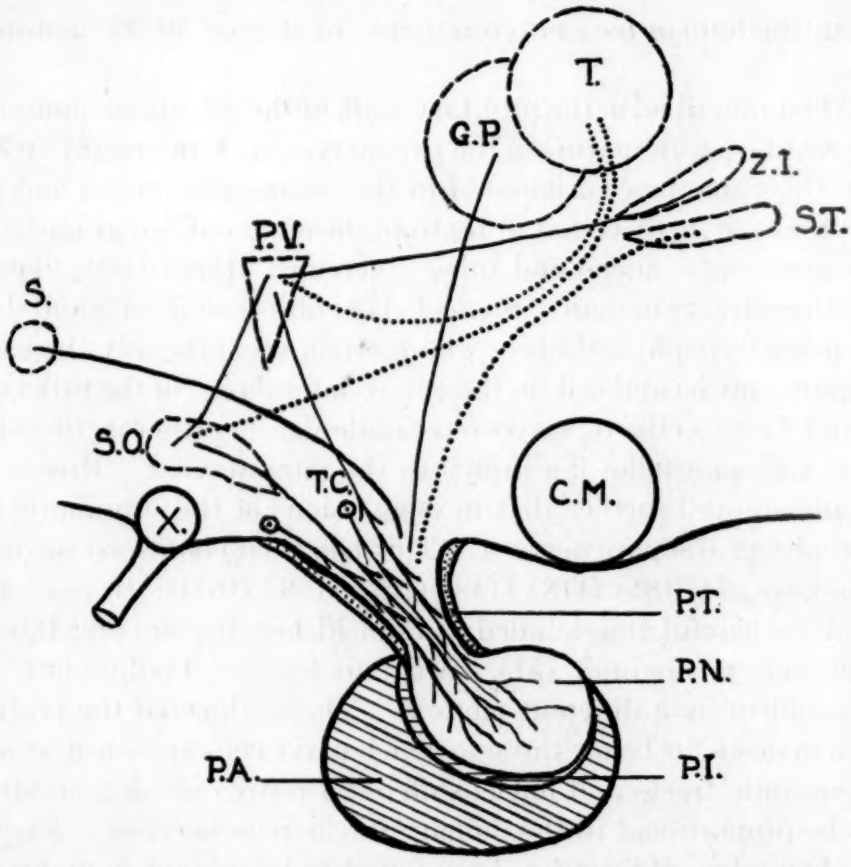


**Fig. 1. Diagrammatic representation of some of the nerve paths afferent to the hypothalamus and pars nervosa.**

***C.M.*, mammillary body.; *G.P.*, globus pallidus; *P.A.*, pars anterior; *P.I.*, pars intermedia; *P.N.*, pars nervosa; *P.T.*, pars tuberalis; *P.V.*, paraventricular nucleus; *S.*, nucleus in septum pellucidum; *S.O.*, supra-optic nucleus; *S.T.*, subthalamus; *T.*, thalamus; *T.C.*, tuber cinereum; *X.*, optic chiasma; *Z.I.*, zona incerta.**

**Dotted lines, periventricular fibres.**

tracts together with the pars nervosa appear to be a single functional unit since injury to any one part of the system results in a possibly proportional atrophy of the other parts; the same may be true of the paraventricular nuclei and their pituitary connections. The hypothalamic nuclei are, in their turn, linked with subthalamic and thalamic nuclei and through these with the sensory and motor systems and all parts of the cerebral cortex. The posterior lobe is also supplied with sympathetic and possibly parasympathetic fibres. Thus, there are many possible routes for the nervous control of posterior lobe function. The efferent

pathways from this area need not be considered here. Some of the work on which this general statement is based is described below. For a fuller account of the literature the reader is referred to (51) (89) (151) (184).

Krause (104) was the first to describe nerve fibres and endings in the pars nervosa. Croll (32) remarked on the abundance of the nerve fibres in the pars nervosa and suggested that as their number appeared to be excessive in proportion to the blood vessels present, their function was secretory rather than vasomotor. Bucy (18) reported the presence of end-bulbs at the terminations of the nerves. This was confirmed in cats by Hair (70). Rasmussen (155) has shown that in the human pars nervosa there are at least 50,000 non-myelinated nerve fibres.

Cajal (23) first described in the pituitary stalk of the rat a thick non-medullated plexus of nerve fibres terminating in the pars nervosa. Greving (67) (68) showed that in man this same tract originated in the supra-optic nuclei and passed to the pars nervosa, and further, that fibres from the paraventricular nuclei travelled to both the supra-optic nuclei and tuber cinereum. Pines (150) likewise drew attention to these tracts in man. Stengel (178) observed in cats and dogs, these and certain other hypophyseal fibres whose origin was probably the caudal part of the supra-optic nuclei and cells in the antero-lateral part of the tuber cinereum. Kary (98) and Lewy (110) reported retrograde degeneration in the supra-optic nuclei of dog and man following injury to the pars nervosa. Broers (15) confirmed this and showed further that in dogs lesions of the supraoptic nuclei resulted in atrophy of the pars nervosa. This has been confirmed for man, dogs, cats and monkeys (71) (81) (118) (148) (157) (158) (161). In particular must be mentioned the careful and detailed work of Fisher, Ingram and Ranson, fully described in their monograph (51). Mogilnitsky and Podljaschuk (133) attacked this problem by a different method. They subjected the posterior lobe to irradiation in doses far below those to which nerve cells are sensitive and found that the supra-optic tracts and nuclei suffered a retrograde degeneration which appeared to be proportional to the damage to the pars nervosa. A comparable supra-optico-hypophyseal tract has been found to be present in many groups of vertebrates (33) (96).

The paraventricular nuclei also are closely linked with the pars nervosa. Greving's (67) observation of fibres from these cells to the supra-optic nuclei and the pars nervosa has already been mentioned. Pines (150) confirmed this finding. Clark (30) reported the existence of similar nerve paths in man. Biggart and Alexander (9) produced lesions with the coagulating diathermy in the anterior hypothalamus of dogs and observed that in those developing diabetes insipidus there was some loss of paraventricular cells. This was confirmed in rats by Rasmussen (156) but not in dogs when the lesion was limited to the stalk or processus infundibuli. Others have found that in dogs, provided the pars nervosa removal be nearly complete, there is a loss of paraventricular cells (81) (148). Rasmussen and Gardner (157) observed some loss of paraventricular cells in man five months after stalk section.

There are afferent paths to the hypothalamus from the thalamus, particularly from the dorsal thalamic nuclei, and the subthalamic nuclei. Woollard (196),

following lesions in the subthalamus of a cat, could trace degenerated fibres into the hypothalamus. The periventricular fibres appear to form a two-way link between the thalamus and the hypothalamus (31) (90) (162). Certain of these periventricular fibres terminate in the supra-optic and paraventricular nuclei (31) (116). Greving (65) described a tractus pallido-hypothalamicus but was uncertain of its exact termination. Degenerative changes have been reported in the globus pallidus of a man who had had diabetes insipidus (8). Loo (113) observed a tract from the zona incerta, some of whose fibres ended in the paraventricular nuclei, and also noted a well-marked septo-hypothalamic tract whose exact termination could not be seen. Through these thalamic and subthalamic centres all regions of the cerebral cortex are linked with the hypothalamus (55) (56) (129) (130) (131) (132) (190).

There is also a sympathetic supply to the pars nervosa which originates from the plexus surrounding the internal carotid artery (36). Brooks and Gersch (16) removed the superior cervical ganglion in young rats and could find no change in the fibre pattern of the pars nervosa following this procedure. Hair (70), on the other hand, found some loss of fibres following cervical sympathectomy in cats and put forward the suggestion that the surviving fibres were parasympathetic in origin.

EXPERIMENTAL AND CLINICAL OBSERVATIONS. This mass of anatomical evidence is meaningless unless experiment and observation show that these pathways are used, and in what direction. There are a number of conditions which may bring about a change in rate of renal water excretion due either sensibly or presumably to an inverse change in the quantity of ADH released from the gland. These conditions will be listed and the evidence considered for and against the hypothesis that the central nervous system is responsible for controlling the liberation of the ADH. i.e., that the hypothalamic-hypophyseal tract is efferent. The conditions which bring about a change in the rate of excretion of water by the kidneys are as follows: 1, lesions of, or operative interference with, the posterior lobe, the nerve fibres passing to the pars nervosa, or the cells of origin of these fibres; 2, direct stimulation of nerve fibres connected with the pars nervosa; 3, exercise; 4, emotion; 5, conditioned stimuli and hypnotic suggestion; 6, certain sensory stimuli; 7, the administration of certain anaesthetic and narcotic drugs; 8, dehydration; 9, hydration.

1. *Lesions of, or operative interference with, the posterior lobe, the nerve fibres passing to the pars nervosa, or the cells of origin of these fibres.* Frank in 1910 (52) first drew attention to the clinical evidence involving the posterior lobe of the pituitary in the production of diabetes insipidus, and three years later Camus and Roussy (24) demonstrated that a lesion of the hypothalamus that did not include the hypophysis also led to the appearance of diabetes insipidus. Since at this time Cajal's observation on the innervation of the pars nervosa had not been properly grasped, these apparently contradictory results led to considerable confusion and argument. Later anatomical studies drew attention to the close link between the hypothalamus and hypophysis and suggested that there was evidence that the opposing hypotheses were in reality compatible.

There have been difficulties in the way of accepting the hypothalamic-hypo-

physeal route for the control of ADH production. It was found experimentally that removal of the posterior lobe alone or section of the stalk (99) (119) (159) did not necessarily result in more than a temporary disturbance of the water balance. Further, simple, or so-called total, hypophysectomy did not lead to the appearance of diabetes insipidus (12) (24) (46).

Fisher, Ingram and Ranson (51) showed that the neural division of the pituitary gland was composed of the pars nervosa, the stalk and the median eminence, and that in all these parts the minute structure was similar and pituicytes could be found. In different animals the anatomical arrangement of the parts varies, but it is evident that all three parts must be removed in order to bring about a complete loss of secreting tissue. This finding appears to dispose satisfactorily of the first of the above mentioned difficulties. The second difficulty, that diabetes insipidus is not evident after "total hypophysectomy" is explained by the observation first of Crowe, Cushing and Homans (34), and later of others, that the anterior lobe has a diuretic function, probably an indirect one (79) (148), and that in its absence a maximum polyuria is improbable or impossible. Once the anatomical arrangement of the posterior lobe tissue and the function of the anterior lobe were better understood most of the contradictions and difficulties were resolved.

Diabetes insipidus can follow posterior lobe removal provided enough of the gland is removed (51). In some species this is not easy. Cushing (35) succeeded in producing intense diabetes insipidus in a dog by the position of a silver clip on the pituitary stalk. Post mortem examination showed a loss of nerve fibres distal to the clip. Fisher, Ingram and Ranson (51) finally demonstrated conclusively that in cats and monkeys the normal activity of the pars nervosa depended on the integrity of its innervation. It has already been mentioned that they had observed that destruction of the supra-optic nuclei resulted in atrophy of the nerve tract to the pars nervosa, and that the pars nervosa itself also atrophied, becoming afibrillar and cellular. Diabetes insipidus resulted in all instances where these changes had taken place to a sufficient degree. These results have been confirmed by others for cats, dogs and monkeys (9) (43) (80) (148) (158). That following the degeneration of the supra-optic nuclei the changes seen in the pars nervosa are of the nature of atrophy is further shown by the fact that no antidiuretic substance can be extracted from such glands (73) (74) (50). Fisher, Ingram and Ranson (51) localised their lesions with great accuracy with the aid of the Horsley-Clarke stereotaxic instrument and showed that the fluid exchange was not affected by lesions in the preoptic area, the lateral and posterior hypothalamus, the mammillary bodies and the tuberal nuclei. In order that diabetes insipidus may be produced the supra-optic cells and tracts must be destroyed nearly completely and bilaterally. In monkeys the water exchange remains normal if 12–16 per cent (117) or 12–14 per cent (157) of supra-optic cells survive operation. The survival of only 5–7 per cent of supra-optic cells is associated with the existence of a marked polyuria (117). In dogs diabetes insipidus is seen provided 85 per cent, or more, of supra-optic cells have atrophied (81) (148). That the survival of such a small part of the hypothalamic-hypophy-

seal system is sufficient to maintain a normal water exchange is not surprising if it be remembered how minute are the doses of posterior lobe extract that can inhibit water diuresis (75) (146) (179).

The literature on the pathological findings in cases of diabetes insipidus in man is enormous and varied, as any lesion of any type encroaching on the hypothalamus or pituitary may lead to the appearance of diabetes insipidus, or it may not. The difficulty here as with all clinical material is that only rarely is the lesion sharply and accurately localised. Furthermore, until recently, the degenerative changes that can occur in a macroscopically normal posterior lobe were not appreciated and therefore not looked for. The same is true of the degenerative changes in the supra-optic and paraventricular nuclei. Many observers believed diabetes insipidus to be purely hypothalamic in origin since it often followed an infection of the meninges which could not directly affect the pars nervosa. For example, Lhermitte (111) (112) describes how diabetes insipidus may result from syphilitic basal meningitis in which at autopsy degenerative changes could be found in the hypothalamic nuclei whilst the posterior lobe appeared to be normal. Others (62) (65) believed that destruction of the pars nervosa, as by metastases, was the cause of diabetes insipidus. Others again (47) (48) (100) believed that the hypothalamus and hypophysis must be considered a single functional unit. Perhaps the most carefully observed and striking cases in favour of the hypothesis of the neuronal control of the posterior lobe secretions are those described by Biggart (6) (7) (8). He found that diabetes insipidus could follow complete destruction of the pars nervosa or interruption of the stalk with resultant loss of cells in the supra-optic and paraventricular nuclei, and also followed lesions of the hypothalamus involving the supra-optic nuclei with secondary atrophy of the pars nervosa. In one case of 30 years' duration the histological appearance of the shrunken and atrophied pars nervosa and the marked loss of supra-optic cells was in every way comparable with the findings of Fisher, Ingram and Ranson (51) after experimental destruction of the supra-optic nuclei in cats and monkeys. In their case Davison and Selby (38) describe similar autopsy findings.

In man, as in animals, diabetes insipidus may follow stalk section. Dandy (37) reported the case of a young woman whose pituitary stalk was sectioned at operation and who promptly developed a permanent diabetes insipidus. As this case was still alive at the time of writing (11 years after operation) it was not known what percentage of supraoptic cells had atrophied. In a man suffering from malignant hypertension the pituitary stalk was divided. This man did not develop diabetes insipidus. Five months after operation he died and at the autopsy it was found that about 15 per cent of his supra-optic cells appeared to be normal (157). This suggests that in man, as in monkeys, the survival of a small proportion of the total number of supra-optic cells, and presumably, therefore, of a similar proportion of pars nervosa tissue, is sufficient to prevent the onset of diabetes insipidus.

That there can be a polyuria of central origin associated with atrophy of the pars nervosa which is not controlled by the administration of posterior lobe ex-

tract (6) (86) (87) (160) (174) is no disproof of the hypothesis that the hypothalamus controls the release of the antidiuretic hormone. The type of diabetes insipidus resistant to posterior lobe extract has yet to be produced experimentally and analysed. The origin of the resistance to antidiuresis is unknown. It may be due to some factor which counteracts the action of posterior lobe extract. Such a factor might be a central nervous irritation disturbing the renal circulation. In the cases described by Snell and Rowntree (174) and Roehm (160) there was an obvious disturbance of the peripheral circulation, and in one of the cases reported by Hoff and Wermer (86) posterior lobe extract became effective when the cerebral pressure was reduced.

It may be seen that observations of the type described in this section offer considerable evidence for the belief that the release of the ADH and the functional existence of the pars nervosa depend upon the integrity of the nerve connection between the gland and the supra-optic nuclei and that when this connection is severed the pars nervosa atrophies and its loss of function is made evident by the appearance of polyuria.

2. *Direct stimulation of nerve fibres connected with the pars nervosa.* It has been reported (27) that on stimulating the central end of the vagus to the isolated viviperfused head of a dog there was an increase in the rate of urine flow from the kidneys of the perfusing body. The urine collected during stimulation was tested on rats and was found to contain an antidiuretic substance which was absent on repetition of the experiment after hypophysectomy. It was concluded that these observations demonstrated the release of the ADH from the pars nervosa on stimulation of the central end of the vagus. The response fatigued but showed recovery if the time interval between stimuli was lengthened (29). This was correlated with exhaustion of the secretory granules in the pituicytes. The functional significance of this reflex is obscure, as is the site of the peripheral sensory receptors.

A slight increase in the rate of urine flow on stimulating the superior cervical ganglion of cats anaesthetised with ether and urethane has been described (168). As this increased rate of urine flow was observed after high cord section it was believed to be of pituitary origin.

Evidence that the pars nervosa is under the influence of the central nervous system has been offered by Karplus and Pecznak (97) and Haterius and Ferguson (78). The former stimulated the hypothalamus in etherised cats and reported that they could thereafter detect the oxytocic and melanophore hormones in the cerebrospinal fluid and in the blood. These results need confirmation. Haterius and Ferguson stimulated the stalk region and noted a resultant increase in uterine activity similar to that produced by the injection of pitocin and unlike the response of the uterus to adrenalin.

There is evidence that stimulation of the stalk can bring about the release of ADH also. Ingram and Barris (91) found that stimulation of the junction of the pars anterior and pars tuberalis resulted in etherised cats in a diuresis. Haterius (77) also applied direct electrical stimulation to the pituitary stalk. He used rabbits under light chloralose and urethane anaesthesia and noticed

that the stimulation led to an inhibition of a pre-existing water diuresis. No inhibition appeared if 2–4 days earlier the pituitary stalk had been transected between the gland and the point of application of the stimulus. Using a different approach Boyd, Lee and Stevens (13) describe what they believe to be a reflex liberation of ADH on stimulating with flashes of light the eyes of unanaesthetised rats. This stimulation caused a diuresis in the rats and did not inhibit an already existing water diuresis. They believed that the diuretic effect depended on the amount of hormone liberated, as on injecting posterior lobe extract in doses equivalent to the amount found in a single rat pituitary gland they obtained similar results. Smaller doses of posterior lobe extract had an antidiuretic effect.

Obviously it is difficult to perform experiments of this type under normal conditions, but, allowing for this limitation, the results do offer evidence for, and not against, the neural control of ADH production.

3 and 4. *Exercise and emotion.* Mackeith, Pembrey, Spurrell, Warner and Westlake (115) noticed that exercise inhibited water diuresis and similar results were obtained by others (41) (101) (193) (195). This inhibition occurs as much in the denervated as in the innervated kidney (101) and persists after the division of both splanchnic nerves (101). It is unlikely, therefore, that it can be due to renal vasomotor changes of central origin or to the liberation of adrenalin. The similarity of the time course of the inhibition due to exercise and that due to injection of posterior lobe extracts has been described (101) and it was suggested that the ADH was responsible for the inhibition due to exercise. That the magnitude of the inhibition due to exercise appeared to be independent of the amount of work done was noticed by all the observers mentioned. In one of Dobreff's experiments it may be seen that in prolonged exercise there was an early and profound inhibition of diuresis, but that recovery set in whilst the exercise was still proceeding (41). In the experiments of Mackeith et al. it was shown that the inhibition appeared early during prolonged exercise and, therefore, could not be due to increasing loss of water through the skin and lungs. The variability of the inhibitory effect of exercise on the excretion of water suggested to Rydin and Verney (164) that the cause must lie elsewhere than in the exercise itself, and in view of observations that emotion similarly inhibited diuresis they set out to find whether this was the real origin of the inhibition of exercise.

Bernard (5) was the first to draw attention to the fact that emotion could inhibit the flow of urine. He ascribed this inhibition to renal vasomotor changes, but any possible participation of the renal nerves in the inhibition has been excluded by showing that it can occur in the dog after the separation of all organic connection between the animal and its kidneys (180). It seemed, then, that a humoral agent must be responsible for the effect observed. The time course of the inhibition resembled that due to the administration of appropriate doses of posterior lobe extract.

Using dogs exercised on a controlled running platform Rydin and Verney (164) showed that mild exercise begun during the height of water diuresis pro-

duced within two minutes a prolonged fall in the rate of urine flow, but that if the dogs were repeatedly exercised under similar conditions the inhibitory response diminished and was finally extinguished. If at this stage of acclimatisation an emotional stimulus, in this case a cacaphonous noise, was simultaneously introduced the inhibition reappeared. They further observed that the time relationships of the inhibition were the same whether it were initiated by exercise or emotion. They then applied only an emotional stimulus during water diuresis and once again observed the same type of inhibition. They felt that these observations justified the belief that mild exercise depended on its emotional content for any inhibitory effect seen. The course of the antidiuretic response to emotional stress was unaltered by successive operations comprising the division of the renal nerves, removal of the right suprarenal gland and denervation of the left, and decentralisation of the whole abdominal sympathetic system with removal of ganglia L2-S1 inclusive. Nor did the inhibition of the urine flow show any parallelism with blood pressure changes simultaneously recorded. When blood pressure changes local to the kidney were induced the urine flow followed these closely. Lastly they showed that the inhibition of urine flow following the injection of appropriate doses of adrenaline were sharp and fleeting and in no way resembled that due to exercise, emotion or posterior lobe extract. This evidence all pointed to the existence of a common cause for the inhibition of water diuresis due to exercise and emotion and indicated that this common cause was not adrenaline.

O'Connor and Verney (143) offered more direct evidence that the posterior lobe of the pituitary is concerned in the emotional inhibition of urine flow. They found that in dogs after removal of the posterior lobe the inhibitory response to an emotional stimulus was either greatly reduced or abolished. Since they were removing the posterior lobe alone it was to be expected (51) that some antidiuretic activity should still be present. O'Connor and Verney determined the quantity of posterior lobe extract that had to be injected in order to produce the degree of emotional inhibition observed both before and after operation. In one dog before operation 5–10 milliunits posterior lobe extract gave the same degree of inhibition as the emotional stimulus. After operation the average dose required was only 0.2 milliunit. There was apparently a functional residuum of 2–4 per cent of the total posterior lobe in this dog.

In a few unpublished observations Pickford attempted the assay of the ADH content of dogs' urine collected during the inhibition caused by a just resented electrical stimulation applied to the flank. The urine was concentrated by the method described by Gilman and Goodman (63) and assayed by Burn's (19) rat method. The pooled urine from four periods of stimulation inhibited diuresis in the rats to about the same extent as did 5 milliunits of posterior lobe extract. After removal of the posterior lobe from the dogs pooled urine from six periods of stimulation had no inhibitory effect on water diuresis in the rats.

Emotional stress can give rise to a polyuric condition resembling diabetes insipidus, but different in that it is curable by suggestion (120).

The emotional inhibition of water diuresis can be mediated by other agents

than the ADH (72). Such inhibitions persist after hypophysectomy or stalk section, are accompanied by a decrease in the renal creatinine clearance and are seen only in certain individuals. These observations do not invalidate the conclusions to be drawn from the experiments described in this section, namely, that emotional stimuli bring about a release or retention of the ADH. We do not know whether the central nervous stimulation is conveyed direct to the pars nervosa or whether changes are first induced in the water balance of the tissues and blood.

5. *Conditioned stimuli and hypnotic suggestion.* It has been shown that an appropriate stimulus of the central nervous system can induce a water diuresis in the absence of the ingestion of water (88) (108) (123). In one series of experiments it was suggested to patients under hypnosis that they had had a drink of water and they were given an empty glass to hold. Shortly after recovery from hypnosis the rate of urine flow increased, the form and time course of this increase being similar to those in control experiments in which water had been ingested without hypnosis. There was a reduction in the specific gravity of the urine and in the concentration of the blood (123). It has also been described how in one dog out of the four that were in use at the time water diuresis appeared in response to the conditioned stimulus of a musical note (126).

Others (4) (21) (22) (66) (69) have recorded similar findings, using as the conditioned stimulus the introduction of the rectal catheter by which the water was usually administered, or the mere placing of the dog in the position and environment in which the unconditioned stimulus was repeatedly given. This diuresis and its inhibition on the approach of a cat are, in part at least, of hormonal origin since they were present to an equal degree in both kidneys even after the denervation of one kidney.

There is no information as to the means whereby this conditioned diuresis is mediated, whether the higher centres, using the nerve paths which undoubtedly exist, induce a direct inhibition of pars nervosa activity, or whether the effect is first on the tissues and blood and only secondarily on the pars nervosa.

Certain observations show that it is possible to set up a conditioned inhibition of an established water diuresis (42) (179). It was found, as will be described later, that in the dog inhibition of diuresis resulted from the insertion and movement in the lumbar area of a needle. It also became evident that this inhibition of water diuresis was more readily caused in animals in which the "operation" had been performed more than once. In one dog it was found possible to cause a diminution of the rate of urine flow by making ostentatious preparations for performing the puncture.

6. *Sensory stimuli.* Certain sensory stimuli are effective inhibitors of water diuresis (179). It was found that in dogs the movement for a few minutes of a needle in the lumbar area resulted in a complete inhibition of diuresis lasting for about 40 minutes. This was the case even when the area of skin and subcutaneous tissue had previously been infiltrated with 2 per cent novocaine. On two occasions the mere shaving of the back over the lumbar region was sufficient to inhibit diuresis. Shaving hair from the leg prior to an intravenous injection

was never observed to lead to an inhibition. The question remains open whether stimulation of the lumbar region is more likely to result in an inhibition than a similar stimulus applied elsewhere.

By denervating one kidney and using the other as a control Theobald and Verney (180) showed that there is no essential difference in the response of the two to the inhibitory influence of afferent nerve stimuli on an established diuresis. That this phenomenon finds its cause in the ADH of the pars nervosa is suggested by the comparison of the inhibitions due to afferent stimuli and to posterior lobe extract, both show the same time lag in onset, the same time course, and after both albumin may be found in the urine.

That the hypothesis of a pituitary cause of the inhibition of diuresis due to afferent stimuli is justifiable has been shown more directly by Haterius (76) (77) who used rabbits under light chloralose urethane anaesthesia and found that in them, as in dogs, stimulation of the lumbar area led to the inhibition of an established water diuresis, but that 2 to 4 days after the destruction of the pituitary stalk lumbar stimulation in no way interfered with the normal rise and fall of renal water loss in diuresis. Further, in 5 out of 8 rabbits stimulation of the pituitary stalk inhibited diuresis, whilst a similar stimulus applied a few days after destruction of the stalk resulted in no inhibition, thus demonstrating that the nerve paths in the stalk must be intact for the antidiuretic action to be apparent, i.e., for the ADH to be liberated in response to nerve stimulation.

7. *The administration of certain anaesthetic and narcotic drugs.* Under the influence of a large number of anaesthetics and narcotics the diuretic effect of water is diminished or abolished (44) (53) (54) (64) (139) (152) (173) (192), though some workers report that drugs such as chloralose and paraldehyde increase the response to water (11) (105) (137) (183). This has not been confirmed by others (44) (179). Bourquin (12) reported that morphine in small doses increased, and in larger doses diminished the renal response to water. The depressant effect of narcotics on water diuresis is probably not a direct action on the kidneys (44), it does not run parallel with that on consciousness (11) (44) (54) (125) or thirst (44), nor is the integrity of the renal or splanchnic nerves essential (53) (114) (183). In anaesthesia there may be a delay in water absorption (84) but that this is by no means the only cause of a reduction in the diuretic response is shown by the fact that the diuresis induced by intravenous or intraperitoneal water (53) (141) is also affected by anaesthetics. The inhibitory effect of these drugs is not due simply to their blood concentrating action (17) (44). It appears, then, that the inhibitory effect of anaesthetics on diuresis must be, in part at least, of hormonal nature. Molitor and Pick (134) (135) (136) (137) postulated the existence of a water controlling centre in the midbrain. This centre they conceived to be under the inhibiting control of the cerebral cortex, and if this restraining influence were removed either surgically or by optimal doses of narcotics such as paraldehyde, the centre would become overactive and water diuresis would be increased, if the centre were depressed then so, also, would be water diuresis. This hypothesis was based almost entirely on experiments on the antidiuretic action of posterior lobe extracts under various conditions. As

proof of the overaction of the water centre under the influence of what they considered a purely cerebral cortical narcotic they reported that paraldehyde rendered null the action of posterior lobe extracts. They believed the posterior lobe extract to act not on the kidneys but on the tissues, either directly, or through the water centre. Their results could not be repeated, in fact, the reverse was found to occur (179). If narcotics by their direct or remote action on a water centre are supposed to counteract the effect of posterior lobe extract at that site, it is difficult to explain how the extract can be an effective antidiuretic in decorticate and decerebrate animals (45) (94) and in anaesthesia (105) (139) (179). If the explanation offered be that the posterior lobe hormone acts on the tissues, then why, in anaesthesia, does blood dilution not result in a diuresis (17) (45) (172)? Further, how does fluid become available for excessive salivation during the inhibition produced by posterior lobe extract, both in the narcotised (179), and in the normal (148) dog, and how can the head of one dog inhibit the urine flow in the isolated perfused kidney of another (187)? The conclusion is inevitable that there is no water centre in the sense postulated by Molitor and Pick, that posterior lobe extract has a direct action on the kidneys, and that in all probability the inhibition of water diuresis seen under anaesthesia is, in part at least, due to the release of increased quantities of ADH. This does not exclude the participation of other factors under certain as yet ill-understood conditions. Thus, in the case of diabetes insipidus described by Frey and Kumpiess (54) the administration of chloral hydrate, trional or morphine reduced the polyuria. Whether there was any surviving normal pars nervosa tissue is unknown. The transitory polyuria of experimentally induced diabetes insipidus begins whilst the animal is still deeply anaesthetised (148). As yet the cause of the transitory phase is unknown. Moreover here as in the previous case, the renal nerves were intact, and the kidney activity may have been modified by centrally initiated vasomotor changes. Bodo and Sweet (10) report that the percental fall in water excretion under morphine and phenobarbital is the same in normal, completely hypophysectomised and in adrenal inactivated liver denervated dogs, and believe, therefore, that neither the pituitary gland nor the adrenals can be involved in the antidiuresis. Here too, the kidneys were not denervated and there is no mention of histological control of the "total" hypophysectomy.

Bruger, Bourne and Dreyer (17) believe that in avertin anaesthesia, despite blood dilution, a fall in blood pressure may be sufficient to account for the antidiuretic action.

Koiwa (102) reports that picrotoxin and $\beta$ naphthylamine despite their known central stimulant action have an antidiuretic effect, which he believed depended on a renal vasomotor effect.

It may be concluded that the anaesthetics and narcotics by their action in modifying the balance of activity in the nuclei of the central nervous system bring about, through nerve channels, an increased release of ADH, thus accounting for the antidiuretic action observed. This does not exclude the possibility that the kidney is also directly influenced through its nerve supply.

8. *Dehydration.* Since the posterior lobe of the pituitary produces an antidiuretic hormone it is a reasonable working assumption that in conditions demanding the conservation of water a greater than normal quantity of ADH should be released. If the conditions be sufficiently stringent it should be possible to show that the gland itself suffers depletion or that fluids likely to carry or receive the hormone contain an increased concentration of it; in other words, dehydration whether due to restricted intake or excessive output should be a strong stimulus to the release of ADH. A number of experiments support this hypothesis.

Using the direct method of assaying the antidiuretic content of the posterior lobe Simon (169) and Simon and Kardos (170) found that in rabbits, guinea pigs and cats after 4–5 days on a dry diet the antidiuretic activity of the posterior lobes was very low compared with controls having an ample supply of water. A decrease in food intake was not responsible for the change since the posterior lobes of guinea pigs and rabbits which had had water ad libitum and no food showed a normal antidiuretic activity. Fighting, work and electrical stimulation had no effect on the hormone content of the gland. Similar findings are reported in rats and dogs (73) (74) (106).

The number and size of the cells in the pars nervosa have been found to vary with the water intake. In rats on a dry diet the cells increased in size and number; if after a period of dry diet water was allowed ad libitum even for one day the pars nervosa was found to present a normal appearance (59) (60).

In searching for the ADH in body fluids certain difficulties are encountered. First, that as we do not know the chemical nature of the principle assays must be biological. The methods of assay are based on the fact that posterior lobe extracts inhibit an induced water diuresis and the polyuria of diabetes insipidus. Dogs (138) (143) (145), mice (61) (140), and rats (19) have been used as test animals. The rat method of Burn (19) or a modification of it is that most commonly used. The second difficulty is that in the higher mammals the kidney is sensitive to minute amounts of the hormone (75) (101) (146) (165) (175) (179), consequently under normal conditions it is probable that the quantities present in the body fluids will be difficult of detection. Thirdly, the body can rapidly inactivate administered posterior lobe extracts (85) (95) and, probably also, therefore the normally released hormone. However, it has been shown (95) that, in terms of the pressor fraction, about 30 per cent of injected posterior lobe extract can be recovered in the urine where it is relatively stable. Using this fact, and starting with the assumption that the ADH is an important factor in the regulation of the volume of the body water Gilman and Goodman (63) argued that following dehydration the level of the ADH in the blood should reach a concentration sufficiently high to allow it to be excreted in detectable amounts in the urine. In support of this argument they found that the urine of rats subjected to dehydration contained appreciable amounts of ADH and that this urinary antidiuretic substance seemed, from its chemical properties, to be identical with that which can be extracted from the posterior lobe. They compared the antidiuretic activity of the urine of normal freely hydrated rats, normal rats

dehydrated either by deprivation of water or the administration of hypertonic NaCl, and similarly treated hypophysectomised rats. There was no detectable antidiuretic activity in the urine of the hydrated animals. Dehydrated normal rats during the time of collection excreted the equivalent of approximately 100–200 milliunits of antidiuretic substance. Hypophysectomised rats' urine, despite the fact that in the time allowed these animals excreted more urine and were therefore more dehydrated than normals, contained no ADH. These results have been confirmed for rats (14) (122) and dogs (74).

Ingram, Ladd and Benbow (92) (93) found that there was an appreciable quantity of ADH in the urine of normal dehydrated cats but that none could be found in the urine of cats, whether hydrated or dehydrated, made diabetic by the interruption of the supra-optic tracts. They further showed that the kidney of a diabetic animal could excrete injected posterior lobe extract, thus ruling out the possibility that no active substance can be found in the urine because the kidney has become incapable of excreting it.

Marx (124) searched the blood for the presence of the ADH and believed that he had demonstrated that the blood of thirsting dogs contained an antidiuretic substance which was absent from the blood of hydrated dogs during diuresis. Marx and Schneider (127) reported the presence in the blood of normal men, but not in the blood of a case of diabetes insipidus, of an antidiuretic substance. Melville (128) found an antidiuretic substance in normal human and dog blood, but did not feel justified in identifying it positively with the hormone of the posterior lobe. The work of Marx has been criticised (188) on the grounds that the circulation in the blood of the described amounts of ADH would, considering the great sensitivity of the human and dog kidney, result in a permanent inhibition of urine flow. There is a possible explanation of the relatively large amounts of ADH found by Marx (82) (109). It has been shown that posterior lobe extracts after addition to rabbits' blood will not pass through an ultrafilter, i.e., the hormone appears to suffer adsorption by one or more of the blood colloids. It has been suggested that the ADH is held in the blood in an inactive state, to be released on reaching the renal tubules. This certainly might explain the presence in the blood of the hormone in amounts greater than the minimum inhibitory dose, but leaves open the method whereby only that amount of the hormone appropriate to the circumstances is released in the kidney.

That there are possible sources of antidiuretic substance other than the posterior lobe has been shown (1) (167) (181). This fact is intrinsically interesting and shows that caution must be used in identifying any particular antidiuretic substance with that derivable from the posterior lobe, but it does not invalidate the conclusions to be drawn from those experiments where antidiuretic substance was absent from the urine after, though present before, hypophysectomy. Walker (191) makes the criticism that hypophysectomy, by a secondary effect on other endocrines, may disturb the production or release of those antidiuretic substances whose origin is elsewhere than from the pituitary. This criticism is rendered invalid by the observation that the antidiuretic substance disappears from the urine after interruption of the supra-optic tracts (93), an operation

which, as far as is known, does not have an effect on any gland except the pars nervosa.

The foregoing experiments give ground for believing that dehydration is a stimulus to the production and release of the ADH of the pars nervosa and that this stimulus is transmitted through the supra-optico-hypophyseal nerves.

9. *Hydration.* If dehydration be a powerful stimulus for the release of the ADH with the object of conserving water, then hydration may be expected to inhibit the release of the hormone. Thus, on Verney's hypothesis (101) (188) an acute water diuresis is due to a temporary reduction in the amount of ADH liberated, and the delay in time between the peak changes in the water load and in the renal excretion rate is related to the speed of destruction of existing ADH and of depression of pars nervosa activity. If this be the case, then after loss of pars nervosa tissue sufficient to lead to diabetes insipidus the maximum urine flow rate might be expected to be quicker in onset and greater in degree. Klisiecki et al. (101) give figures in a normal and a diabetic man of the period in minutes between the midtime of drinking and the first increase in the rate of urine flow. In the diabetic even when thirst was present, which must be supposed to mean that there was still some degree of dehydration, this period was shorter than in the control. In a dog which had had the whole pituitary gland removed and the supra-optic tracts sectioned and in which at post mortem only about 2 per cent of surviving supra-optic cells were found, the rate of urine flow in diuresis was consistently higher after as compared with before operation (148). That in experimental diabetes insipidus the maximum rate of urine flow in water diuresis is frequently no higher than in normal animals does not invalidate the pituitary hypothesis, since such minute quantities of posterior lobe extract are effective in altering the rate of urine flow, that even a small amount of surviving pars nervosa tissue may be sufficient to ensure a normal water diuresis (75) (179).

Fee (45) and Newton and Smirk (142) from their observations came to the conclusion that neither the hypothalamus nor the pars nervosa was of primary importance in the production of polyuria and water diuresis.

They based this belief on the results of their observations on the water excreting capacity of decerebrate and totally hypophysectomised cats and dogs. They found that, allowing time for the excretion of the volatile anaesthetic used in the preparatory stages of the experiments, such animals could excrete ingested water in an apparently normal manner, there was the normal interval of time between the absorption of the water and the onset of diuresis, the maximum rate of urine flow occurred at about 80 min. after ingestion ended, and thereafter the urine flow rate fell away in a normal manner. They failed to observe a true persistent polyuria. Their results were only in part confirmed by Tsai (182).

A possible explanation of these results is afforded by an examination of the figures Fee gives (45). Using dogs he administered as much as 350 cc. water and the maximum urine flow rate attained was 22.3 cc. per 20 min. After 3 hours, of the 350 cc. given, 122 cc. had been excreted. This low rate and small total excretion can by no means be considered typical of a normal water diuresis. The weight of the dogs is not given, but experience with unanaesthetised dogs of

about 12 kgm. shows that after 300 cc. of orally administered water a maximum urine flow rate of at least 60 cc. per 20 min. may be expected, and that in 3 hours about 85 per cent, or more, of the volume given has been excreted by the kidneys. On the other hand, after removal of the whole pituitary gland an unanaesthetised dog has, after water administration, a maximum urine flow rate that is about half, or less, of the preoperative rate under similar conditions (148) and does not excrete nearly as much as usual in 3 hours. Thus, in these dogs an increase in the water load does result in an increase in the rate of urinary water excretion, but this increase is far below the normal and may persist for a longer time. If a polyuria be present it is small in degree. The poor response of the kidney to water appears to be due to the loss of the anterior lobe of the pituitary. Thus, the diuresis obtained by Fee and Newton and Smirk, whose animals had no anterior lobe, may have been of the type seen in unanaesthetised totally hypophysectomised dogs, where the loss of posterior lobe tissue tends to polyuria and increased diuresis, but the loss of anterior lobe tissue counteracts these (79) (148). Nor is it justifiable to assume, as Newton and Smirk did, that the capacity of the kidneys to exhibit a water diuresis is evidence of their capacity to exhibit a spontaneous polyuria. These two capacities can vary independently of each other. An unanaesthetised dog, at a time early after operation, when it is polyuric, may be incapable of producing a normal water diuresis (148). The observations of Fee and Newton and Smirk, then, are no disproof of the existence, under normal conditions, of a decreased liberation of ADH in response to the ingestion of water. Moreover, it has never been suggested that, in any given conditions, the ADH is the only factor limiting the rate of urine flow.

Hart and Verney (75) found that the fall in the rate of urine flow produced by pituitary extracts in the course of a maximum water diuresis was smaller when the extract was given early than when given late. Pickford (146) in a series of experiments planned to discover whether a simple relationship existed between the degree of inhibition in the rate of urine flow resulting from the administration of pituitary extract on the one hand, and the amount of tissue water in excess of the normal on the other (the water load), found that there was a roughly inverse proportionality between the two. Thus, when as little as 0.00002 cc. (0.2 milliunit) posterior lobe extract was injected intravenously into dogs early during the course of a water diuresis the resultant inhibition was considerably less than when the same dose was given later in diuresis. This difference in response to the extract may easily be explained if it be assumed that with high water loads the production or release of the naturally occurring hormone is inhibited. At such a time an administered dose of extract would be added to a small quantity, or none, of the spontaneously produced hormone and would be, therefore, relatively ineffective. As the water load falls increasing quantities of the natural agent would be present in the blood and the injection of exogenous hormone would result in a greater effective concentration and, hence, of a greater degree of inhibition than previously.

It would appear that when the water load of the body is high, the liberation of the ADH from the pars nervosa temporarily ceases until such time as the

water load falls to normal levels. When the body is dehydrated there is an increased circulation of ADH in order that water may be retained by the kidneys and the osmotic balance of the blood and tissues preserved. In dehydration the posterior lobe activity probably varies in response to nerve impulses. Until the contrary be proved, it would be needlessly complex to suppose that whilst the posterior lobe is stimulated by a neuronal mechanism it is inhibited by some other means. Moreover, in all the previous sections it has been shown that the balance of evidence is in favour of the regulation of the antidiuretic activity through the hypothalamic-hypophyseal tracts.

It remains to be considered by what means the nerve impulses are transmitted.

*Transmission of the stimulus.* It is probable that the transmission of the stimulus to the posterior lobe depends on the release of acetylcholine at the neuronal or neuro-glandular junction. It has been shown that acetylcholine injected into the ventricles of cats produces effects similar to stimulation of those parts (39) (40). Others have described how on stimulation of the central end of the vagus a cholinergic substance, probably acetylcholine, was released from the central nervous system. This substance was believed to originate in the hypothalamus (25) (26). During the time of vagal stimulation a pressor and an antidiuretic substance were described as being present in the jugular blood, provided that the hypophysis was present (28), i.e., acetylcholine liberated in the hypothalamus by stimulation of the vagus caused the release of posterior lobe hormones into the circulation. Pickford (147) showed that in the normal conscious atropinised dog intravenous acetylcholine inhibited a water diuresis. Blood pressure records taken before and during the response to acetylcholine showed that it was improbable that the prompt and brief blood pressure changes could be concerned in the delayed and prolonged inhibition of urine flow. Nor could adrenaline be the causative agent as this substance given intravenously produces a sharp, transient and synchronous change in the blood pressure and the urine flow. Further, others have shown (164) (166) (189) that vasomotor changes localised to the kidneys bring about changes in the rate of urine flow whose time course closely follows that of the vascular changes. Denervation of the kidneys did not abolish the response to acetycholine. As the time course of the inhibition and the changes in chloride concentration of the urine were similar to those seen after administration of posterior lobe extract, and since acetylcholine, like posterior lobe extract produced a diuresis in the thirsting unanaesthetised dog, it seemed probable that the inhibitions were causally related. If indeed that were so, then loss of the pars nervosa should abolish the response to acetylcholine. This was found to be the case. After removal of the posterior lobe neither the antidiuretic nor the diuretic effects of acetylcholine could be elicited, nor the rise in the rate of chloride excretion.

A few observations (149) both before and after removal of the posterior lobe have been made on the antidiuretic content of the urine excreted during the inhibition of diuresis produced by acetylcholine. The methods of Gilman and Goodman (63) and Burn (19) were used. It was found that before operation the pooled urine from 4–6 periods of acetylcholine administration resulted in an

inhibition of diuresis in the rats, whereas after removal of the posterior lobe similarly collected urine did not inhibit the rate of excretion in the rats. These findings all point to acetylcholine as the transmitter of the impulse to the pars nervosa, but give no information as to its site of action, whether at the hypothalamic synapses or at the nerve terminations in the posterior lobe.

## CONCLUSIONS

The release into the circulation of the ADH made in the pars nervosa appears to be under the control of the central nervous system. The impulses travel to the pars nervosa along the hypothalamic-hypophyseal tracts, and somewhere on this route depend on acetylcholine for their transmission. The primary stimulus which initiates this reflex is not known with certainty. The receptors for this stimulus are central and not peripheral.

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# PRESENT VIEWS ON THE MODE OF ACTION OF ACETYLCHOLINE IN THE CENTRAL NERVOUS SYSTEM

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The conception that acetylcholine acts as chemical transmitter in the central nervous system is a logical extension of the theory of chemical transmission by acetylcholine across ganglionic synapses and from motor nerve endings to the motor endplates of skeletal muscles. About ten years ago Dale (1934), when discussing the evidence for the transmission by acetylcholine across ganglionic synapses, pointed out that this theory involved a much wider aspect, viz., a similar transmission at central synapses. A few years later, in his Harvey Lectures (1936/37), he took up the problem again. He drew attention to the fact that Eccles had used the sympathetic ganglion "as furnishing an accessible model of the synapses of the central grey matter" and that Sherrington had looked upon the transmission at a motor endplate "as probably furnishing a pattern, or paradigm, of what happened at a central synapse." It is thus not surprising that the methods of investigation used for the ganglion and for voluntary muscle should be extended to the central nervous system.

The following three lines of research have supplied the main evidence on which the acetylcholine theory in the peripheral nervous system is based: (1) a study of the effects of acetylcholine and of drugs which, like atropine or nicotine, may influence the response to acetylcholine, (2) a study of the effects of eserine or other inhibitors of cholinesterase on the response to nervous impulses and (3) experiments which show that acetylcholine is in fact liberated during nervous activity. Supporting evidence has been obtained from determinations of the acetylcholine and cholinesterase contents of a tissue under different conditions and from the presence in nervous tissue of an enzyme system responsible for the synthesis of acetylcholine. In the last ten years these lines of research have been applied to the central nervous system and the results thereby obtained are reviewed in the following sections.

1. Central actions of acetylcholine. The peripheral effects of acetylcholine have been classified as muscarine- and nicotine-like actions (Dale, 1914). The effects on smooth muscles, heart and gland cells, like the similar effects of muscarine, are sensitive to atropine, whereas the effects on autonomic ganglia, the medullary cells of the suprarenals and the motor endplates of mammalian skeletal muscles are not affected by atropine, or at least not in doses which affect the muscarine-like actions, but they are abolished by large paralysing doses of nicotine.

Acetylcholine has both stimulating and depressant actions on the central nervous system and both actions appear to be sensitive to atropine. They could therefore be classed as muscarine actions. It is, however, not advisable to make the atropine sensitivity the sole test for classification; its too strict application would separate even peripheral actions which belong together. For in-

stance, atropine abolishes the effect of acetylcholine on motor endplates in frogs but not in mammals and in larger doses atropine may depress the nicotine action of acetylcholine in other structures (Feldberg, Minz and Tsudzimura, 1934; Feldberg and Vartianen, 1935; Marrazzi, 1939). These limitations do not invalidate the atropine test; but in applying it we have to realize that very large doses of atropine depress cells in an unspecific way so that drugs other than the parasympathomimetic ones become ineffective. The central actions of acetylcholine differ from those of muscarine and other parasympathomimetic drugs. Pilocarpine, for instance, stimulates vegetative centres (Cushing, 1931) but, unlike acetylcholine, is ineffective when applied to the cerebral cortex (Miller, Stavraky and Woonton, 1940).

The central actions of acetylcholine are best viewed together with those on motor endplates and on autonomic ganglia. They have this in common: the changes brought about at nerve cells or endplates initiate a wave of excitation propagated along a nerve or muscle fibre. If the acetylcholine persists at the nerve cell or endplate beyond the refractory period of the fibre a new wave of excitation may be initiated and this may be repeated over and over again. Thus the arterial injection of acetylcholine into a muscle causes a tetanic contraction of the muscle fibres; into a ganglion a repetitive firing along the postganglionic fibres. Similar waves of excitation will be initiated from the cells of the central nervous system.

Acetylcholine probably acts in these structures by virtue of its depolarising property which has been demonstrated by Beutner and Barnes (1941; 1942). They found that acetylcholine in minute doses produced electrical negativity when allowed to act on artificial interfaces of water and water-insoluble substances resembling lipoids. A depolarisation of the endplate or nerve cell by acetylcholine might thus constitute the effective stimulus for the initiation of a wave of excitation. In the electric organ of some fishes, which consists of modified motor endplates lacking the contractile muscle fibres, this depolarisation is the final event and the electrical discharge may be looked upon as the sudden depolarisation of the plates, b rought about by the release of acetylcholine from the nerve endings covering their ventral surfaces. In fact acetylcholine injected into such an organ has an electrogenic effect (Feldberg and Fessard, 1942).

When acetylcholine is released in the course of normal nervous activity, it is quickly inactivated, and consequently the cell surface remains depolarised for an extremely short interval, then becomes polarised and ready for the acetylcholine released by the next nerve impulse. But when acetylcholine is applied artificially or released in the presence of eserine, it will persist at the cell surface and the mechanism of depolarisation and polarisation may become deranged. This derangement may manifest itself as a depression of excitability produced by acetylcholine. The fact that under these conditions acetylcholine is predominantly depressant in action might thus find an easy explanation. Against this attractive theory, however, the fact must be stated that atropine antagonises the central depressant actions of acetylcholine and that there is no evidence that atropine is able to counteract, by a polarising action, the depolarising one of

acetylcholine. The conception of acetylcholine as a depolarising agent is useful if applied to the theory of central transmission, but we have to realize that the mode of action of acetylcholine in the central nervous system is as little understood as that of any other drug acting peripherally or centrally.

*Cerebral cortex.* The effects of acetylcholine on the electroencephalogram were first recorded by Sjöstrand (1937). Acetylcholine applied locally to the cortex increased the amplitude of the large diphasic waves produced by strychnine or eserine and caused an increase in the frequency and grouping of the waves. Strong concentrations or repeated applications of acetylcholine completely inhibited the waves. Bonnet and Bremer (1937b) and Bremer (1938) divided the brain stem in cats above the pons in order to avoid the use of anesthetics and to prevent incoming impulses; under these conditions, injections into the carotid artery of 0.1 to 0.2 $\mu$g acetylcholine increased the amplitude and frequency of the Berger waves. The effect resembled that obtained during an awakening reaction. In the acoustic region there was not only increased spontaneous activity but also an increased after-discharge following acoustic stimuli. Larger doses of acetylcholine had a depressant effect. The observations have been confirmed by Moruzzi (1939) for the area of mastication in rabbits. In addition he found that acetylcholine injections decreased the threshold for cortical stimulation, comparable to the "facilitation" obtained on stimulation of a neighbouring area. Miller, Stavraky and Woonton (1940) applied acetylcholine with bits of blotting paper to areas of the motor cortex of cats and rabbits; the result, which resembled a very weak eserine action, was a reduction of the amplitude of the slow waves and it was attributed to asynchronous firing of a large number of neurones. On the previously eserinised cortex acetylcholine caused within 2 minutes large and rapid rhythmical waves composed of diphasic spikes, resembling strychnine spikes, with smaller components superimposed. The spikes were associated with motor effects on the contralateral side (vibrissae movements, increased tremors and in rabbits movements of mastication). Using the same method, Chatfield and Dempsey (1941) observed no change in electrical cortical activity with acetylcholine alone, but when acetylcholine was given after prostigmine, which itself caused bursts, the individual potentials between the prostigmine bursts became spikes, and new spikes appeared. This was not seen in all cortical areas. Later fast low potentials appeared. Potentials resulting from single shock stimulation of afferent nerves were either not affected or slightly reduced. Such stimulation no longer produced the normal sharp localisation of sensory potentials. Potentials appeared also in other cortical areas although not, as with strychnine, on the contralateral side. When the cortex was isolated from the thalamus, only the fast voltage waves remained; therefore these were thought to represent intrinsic cortical activity, the other components representing activity in thalamocortical circuits.

In order to obtain in cats, without eserine or prostigmine, pronounced changes in electrical cortical activity, acetylcholine has to be applied to the cortex in very strong concentrations (2.5 to 10 per cent); acetyl-$\beta$-methylcholine and carbaminoylcholine are also active, the latter in weaker concentrations (0.08 to

0.3 per cent). The effects, which are localised and persist as long as the drugs are kept in contact with the cortex, resemble the electrical changes recorded from the human cortex during convulsive seizures (Brenner and Merritt, 1942). When large doses of acetylcholine are injected intracisternally into cats or man generalised convulsions occur (Fiamberti, 1937; Brenner and Merritt, 1942). Similar effects produced by intravenous injections of very large doses are, however, not necessarily due to central actions of acetylcholine (Rossi, 1939; Harris and Pacella, 1943; Cohen, Thale and Tissenbaum, 1944).

Williams (1941) found that carbaminoylcholine given subcutaneously to epileptics increased the *petit mal* activity in the electroencephalogram, and that acetylcholine given intravenously sometimes caused epileptic outbursts and electrical changes in the electroencephalogram characteristic of *petit mal* activity.

*Sleep.* According to Dikshit (1935) acetylcholine (0.1 to 0.5 μg.) injected into the lateral ventricle of unanesthetised cats, produces a condition resembling sleep lasting for two to three hours. Silver and Morton (1936), in similar experiments, observed only drowsiness in their cats. In unanesthetised monkeys, the intraventricular injection of a few milligrams of acetylcholine had no effect of this kind (Light and Bysshe, 1936) whereas in man it led to sleep in one, and to drowsiness in two, out of eight patients (Henderson and Wilson, 1936).

*Respiration.* An intravenous injection of acetylcholine stimulates, and in larger doses depresses, respiration. Whereas the depression is central in origin, the stimulation, when produced in this way, is mainly due to an action of acetylcholine on the chemo-receptors and not, as assumed by Villaret, Besancon and Cachera (1934), to an effect of secreted adrenaline (Schweitzer and Wright, 1938). In atropinised and eserinised cats with chemo-receptors excluded, the effect of intravenous injections of large doses of acetylcholine is no longer mainly depression of respiration, but strong stimulation preceded by inhibition (Schweitzer and Wright, 1938). In dogs with denervated chemo-receptors Heymans, Boukaert and Farber (1935) observed hyperpnoea, sometimes preceded by inhibition of respiration, after arterial injection of acetylcholine. Much larger doses were necessary than for stimulation of the chemo-receptors. Recently hyperactivity of the respiratory centre after arterial injections of acetylcholine was also observed in dogs by Gesell, Hansen and Worzniak (1942) and by Gesell and Hansen (1943). Subthreshold doses became effective after eserine. The response to acetylcholine was essentially a normal hyperpnoea; it was modified by incoming impulses. Thus acetylcholine augmented the responses to sinus nerve stimulation or to lung inflation. A slow arterial infusion into a dog with denervated carotid bodies produced reinforcement of thoracic respiration, temporary irregularity of "torsal" respiratory movements, associated with subnormal pulmonary ventilation, and initiation or strengthening of the facial respiratory contractions (Gesell and Hansen, 1943).

Dikshit (1934a, b) using intraventricular injection of acetylcholine (0.1–1 μg.) into unanesthetised cats, usually found temporary arrest, but in some cases acceleration, of respiration. Silver and Morton (1936) observed irregularities of respiration under similar conditions in a few cats. Miller (1943) found in-

creased rate of respiration on local application of acetylcholine (1 in 50 millions) to the floor of the fourth ventricle in decerebrate cats. Shuh, Wang and Lim (1936) as well as Gesell, Hansen and Worzniak (1943) observed in anesthetised dogs hyperpnoea on local application of acetylcholine to the floor of the fourth ventricle; in unanesthetised dogs the intracisternal injection of 5 mgm. carbaminoylcholine stimulated respiration but acetylcholine was ineffective even in much larger doses (Weinberg, 1937). In unanesthetised monkeys (Light and Bysshe, 1933) and in man (Henderson and Wilson, 1936) the intraventricular injection of a few milligrams of acetylcholine had no appreciable effect on respiration.

*Vomiting, coughing, sweating, defaecation and micturition.* In man (Henderson and Wilson, 1936) the intraventricular injection of 2.5 mgm. of acetylcholine produced nausea, retching, sweating and often vomiting; the centres for lacrimation and salivation were scarcely affected. Apart from a slight fall in rectal temperature, due probably to sweating, the temperature was not affected. In one instance there was coughing and a feeling of suffocation. Despite the regular occurrence of peristalsis, defaecation and micturition took place once only. The central origin of sweating was particularly striking in a patient with interrupted sympathetic innervation to the left arm and face, where sweating was absent. In some instances the injection appeared only to sensitize the sweat centre, excessive sweating being produced by the stimulus of muscular effort. In unanesthetised cats signs of nausea were observed by Silver and Morton (1936) after intraventricular injections of acetylcholine (20 $\mu$g.). Local application of acetylcholine (1 in 50 millions) to the trigonum hypoglossi beneath the floor of the fourth ventricle caused movements of the tip of the tongue and repeated deglutition (Miller, 1943).

*Pupillary reactions* were not produced by the intraventricular injection of acetylcholine in man (Henderson and Wilson, 1936).

*Bronchi.* In curarised dogs Houssay and Orias (1934) found no central vagal effects on the bronchi after arterial injections of acetylcholine.

*Heart.* Dikshit (1934b) observed that intraventricular injections of 1 $\mu$g. acetylcholine or less into cats under chloralose produced cardiac irregularities similar to those obtained on central vagus stimulation. The centre affected must be above the Sherrington level, probably the hypothalamus, because denervation below the Sherrington level abolished the effect. Silver and Morton (1936) occasionally observed similar cardiac irregularities. According to Gremels (1938) a slow infusion of acetylcholine into the carotid artery of cats increased the excitability of the cardio-inhibitor centre, but the chemo-receptors were not excluded in these experiments. In dogs with eliminated chemoreceptors the arterial injection of a large dose of acetylcholine caused bradycardia (Heymans, Boukaert, Farber and Hsu, 1935). The increase in heart rate observed by Weinberg (1937) in large unanesthetised dogs after intracisternal injection of 90 mgm. of acetylcholine may well have been due to an action on the suprarenal medulla after absorption of the acetylcholine. In unanesthetised monkeys the intraventricular injection of a few milligrams of acetylcholine did not change the heart rate (Light and Bysshe, 1933), whereas in man it produced

sometimes slowing and in one instance acceleration (Henderson and Wilson, 1936).

*Blood pressure.* Heller and Kusonoki (1933) saw no changes in the blood pressure of cats after intracisternal injection of 5 to 10 μg. acetylcholine. Dikshit (1934b) in his experiments with intraventricular injections into anesthetised cats, observed irregular changes and Silver and Morton (1936) observed a small rise followed by a sharp fall; the acetylcholine was injected into the hypothalamic region or into the lateral ventricle. Applied by iontophoresis to the floor of the fourth ventricle acetylcholine caused a rise in arterial blood pressure. This procedure was used as a means of mapping out the pressor area in dogs (Shuh, Wang and Lim, 1936). The pressor effect observed by Weinberg (1937) on intracisternal injection of 90 mgm. acetylcholine into large unanesthetised dogs may have been due to the peripheral actions of absorbed acetylcholine; 5 mgm. carbaminoylcholine, similarly injected, caused intense vasoconstriction but only a slight rise in blood pressure. Haas (1939) denies any central action of acetylcholine on autonomic functions (blood pressure, blood sugar and temperature) because he found it less effective on intracisternal than on intravenous injection. His own experiments were negative but they do not invalidate, as he claims, the results of other workers who used small doses of acetylcholine. The intraventricular injection of a few milligrams of acetylcholine into unanesthetised monkeys produced a long lasting but moderate fall in blood pressure (Light and Bysshe, 1933) whereas in man it had no effect or caused a slight rise in pressure; but there was some flushing of the skin (Henderson and Wilson, 1936).

*Spinal cord: Discharge of motor impulses to skeletal muscles* by acetylcholine was suggested by some observations of Feldberg and Minz (1932). In atropinised decapitated cats large doses caused muscular twitching, mainly of central origin; an apparently similar effect was observed by Lefebre and Minz (1936) when applying acetylcholine on the isolated spinal cord of frogs. Striking evidence for this action was obtained by Bülbring and Burn (1941) in experiments on dogs with separate circulation to the hind limbs and to the spinal cord, the acetylcholine being injected into the latter. Contractions of the limb muscles occurred with 0.06 to 1 mgm. acetylcholine and, when eserine was given previously, with 10 and even 1 μg. In decerebrate cats Calma and Wright (1944) observed widespread contractions after arterial injections of acetylcholine. In man the intrathecal injection of even 500 mgm. acetylcholine was ineffective, but a smaller amount injected with a dose of prostigmine, which by itself would only depress the spinal cord slightly, caused a profound depression (Kremer, 1941).

*Reflexes in somatic nerves* are affected in different ways. Moreover the same reflex may apparently be affected differently in different species and under different experimental conditions, such as anesthesia, mode of administration, dosage, pre-treatment with eserine, etc. Depression is usually obtained more easily than augmentation. In cats under chloralose the intravenous injection of acetylcholine depresses the *knee jerk* and abolishes strychnine convulsions. The depressant effect on the knee jerk was shown to be mainly one on the spinal cord and to be increased after eserine (Schweitzer and Wright, 1937). A slight

transient increase in muscular tone as well as an initial augmentation of the knee jerk was observed but was attributed to indirect circulatory effects. Recently, however, Calma and Wright found a strong increase in tone of the quadriceps muscle in decerebrate cats owing to a central action of the acetylcholine injected arterially. Carbaminoylcholine caused stronger inhibition of the knee jerk than acetylcholine but acetyl-β-methylcholine had no effect at all. Bülbring and Burn (1941) in their experiments on dogs with separated cord circulation occasionally observed an increase of the knee jerk with 20 μg. acetylcholine, but with 2 mgm. it was temporarily depressed and then augmented. After eserine or prostigmine acetylcholine was mainly depressant. When nicotine had abolished the knee jerk large doses of acetylcholine restored it temporarily. The stable choline esters, acetyl-β-methylcholine and carbaminoylcholine, caused initial inhibition followed by augmentation. The *flexor reflex* was found to be depressed in cats by intravenously injected acetylcholine (McKail, Obrador and Wilson, 1939) but slightly augmented in dogs when acetylcholine was injected into the separately perfused cord circulation (Bülbring and Burn, 1941). In the latter experiments depression of the reflex was interrupted by augmentation when acetylcholine was injected after eserine. Unlike the knee jerk, the flexor reflex, however, was never abolished by acetylcholine. The stable choline esters caused augmentation followed by progressive depression.

Bonvallet and Minz (1938b) found that acetylcholine, given intravenously, increased the excitability of the linguo-maxillary reflex. According to these authors (1937a, b; 1938) acetylcholine depresses reflex excitability in spinal animals but increases it in midbrain animals (dogs, cats, rats). The acetylcholine was injected intravenously, or even intraperitoneally, without any precautions to avoid peripheral actions. But similar results were obtained in frogs on application of acetylcholine to the central nervous system: applied to the spinal cord it decreased reflex excitability (Lefebre and Minz, 1936), applied to the midbrain it increased reflex excitability (Bonvallet and Minz, 1938).

Torda (1940) obtained depression of the crossed extensor reflex in spinal toads when they were perfused with strong concentrations of acetylcholine, its peripheral effects being excluded. The depression was counteracted by central stimulation of the intra-abdominal branches of the homolateral sciatic nerve. Reflexes in cats behaved in much the same way (Martini and Torda, 1939) and Bülbring and Burn (1941) obtained similar results in their experiments on dogs with separate cord circulation. After acetylcholine the normal inhibition of the knee jerk, on stimulation of the homolateral posterior tibial nerve, was followed by a large exaggerated rebound.

*Sympathetic spinal centres.* In dogs with transverse cord section the intravenous injection of acetylcholine or of carbaminoyl-β-methylcholine caused constriction of the separately perfused spleen, which was connected with the dog by its nerves only (Farber and Heymans, 1936). The possibility of an action in the sympathetic ganglia was not excluded.

2. CENTRAL ACTIONS OF ESERINE AND OF PROSTIGMINE. Eserine, like acetylcholine, has both stimulant and depressant central effects. If these effects could

be proved to be wholly or mainly due to inhibition of cholinesterase activity they would furnish perhaps the strongest evidence so far available for the acetylcholine theory of central nervous activity. They would prove that acetylcholine is continuously released, that it is normally, either wholly or to a great extent, inactivated by cholinesterase and that the amounts released, when allowed to accumulate, profoundly alter central nervous activity. It could then be assumed that the acetylcholine normally released exerts such functions as are manifest in a more pronounced degree after eserine.

With regard to the *peripheral* actions of eserine it is generally accepted that they are the result of inhibition of cholinesterase. On the other hand if eserine competes with acetylcholine for the same receptor groups of the cholinesterase, it might be expected to compete with acetylcholine in a similar way for the respective functional receptors in gland cells or muscle fibres. Thus the possibility exists of acetylcholine-like actions of eserine independent of the inhibition of cholinesterase. In fact the stimulating action which eserine exerts on some organs when given in extremely large concentrations may be explained in this way. The following facts, however, suggest that the usual peripheral effects of eserine result from inhibition of cholinesterase.

(1) The stimulating action of eserine on smooth muscle is characterised by its long latency, its slow development and its gradual disappearance when the eserine is washed out. These peculiarities point to an indirect mechanism of action.

(2) The smooth muscles of the intestine and of the uterus are both sensitive to acetylcholine but only those of the former to eserine.[1] As a continuous release of acetylcholine and its replacement by synthesis occur in the intestinal wall, probably owing to the activity of the nerve cells of Auerbach's plexus between the muscle layers (references see Feldberg and Solandt, 1942), inhibition of cholinesterase must lead to muscular contractions. There is no such nerve plexus and no evidence for a continuous release of acetylcholine in the uterus, and therefore eserine, if acting solely by inhibition of cholinesterase, should not stimulate the acetylcholine sensitive muscle fibres.

(3) Eserine constricts the pupil, but not when the postganglionic nerve fibres from the ciliary ganglion have degenerated after its removal, although the pupil still contracts to pilocarpine in this condition (Anderson, 1905). As first suggested by Gaddum (1936) acetylcholine is released in small amounts from the endings of the ciliary nerve in close proximity to the muscle fibres and accumulates after eserine; but after nerve degeneration acetylcholine is no longer released and practically disappears from the iris (Engelhart 1931), which remains however sensitive to the direct action of parasympathomimetic drugs.

(4) Changes in the molecular structure of eserine which lead to disappearance of the inhibitory action on cholinesterase also remove the typical miotic properties on the normally innervated pupil (Stedman, 1926).

The assumption that eserine has no direct stimulating peripheral effects leads to new conceptions concerning the function of autonomic ganglia and even of nerve fibres. Eserine slows the heart, even after cutting the vagi, as already shown by Fraser in 1867. The action might result from random impulses travelling down the cut nerve fibres but is more probably due to a continuous release

[1] In very large concentrations eserine also stimuates the uterus muscle (White and Stedman, 1931). The following arguments do not apply to the effects of such concentrations; they may be, as mentioned above, manifestations of a direct action of eserine.

of acetylcholine by impulses originating in the ganglion cells in the wall of the heart. According to this view these cells are weakly automatic centres comparable to the cells of the plexus of Auerbach. These cells retain their ability to release acetylcholine after degeneration of the pre-ganglionic vagus fibres (Goffart and Bacq, 1939). Of particular interest for the problem of central synapses is the action of eserine on motor endplates. It causes fascicular twitching and fibrillation of skeletal muscles even after severance of the motor nerves, but the effect disappears two to three days after nerve section (Langley and Kato, 1914) i.e., at a time when nerve fibres have lost their ability to synthesize acetylcholine (Feldberg, 1943), but the motor endplates have become more sensitive to acetylcholine. The fascicular eserine twitchings may be accounted for by the release of acetylcholine by random impulses passing down the cut nerve (Bacq and Brown, 1937); the fibrillations cannot be explained in this way. They may be due to a continous release and resynthesis of minute amounts of acetylcholine from the endings of the "resting" nerve, so slight as to have no effect in the absence of eserine (Feldberg, 1945). According to this view the motor endplates are always insensitive to the direct action of eserine.

There is thus strong evidence that the peripheral effects of eserine are all acetylcholine effects and that apart from its inhibitory action on cholinesterase eserine is a pharmacologically inert substance. We have dealt in such detail with the peripheral effects because the assumption of a different mechanism for the central effects of eserine would be unnecessary if the conditions for an indirect mechanism of action prevail also in the central nervous system. This is the case. The central actions of eserine resemble those of acetylcholine sufficiently to be explained by the accumulation of the latter. Acetylcholine is present in the central nervous system and apparently is continuously released and replaced by synthesis; the tissue contains cholinesterase, therefore acetylcholine is bound to accumulate and exert its effects in the presence of eserine.

There are, however, certain difficulties. Under apparently similar conditions some inhibitors of cholinesterase may be excitatory, others depressant; even the same substance in the hands of different workers may have opposite actions. A review of the old as well as of the new literature reveals this strange pharmacological irregularity which has led to as yet unresolved controversies. Schweitzer, Stedman and Wright (1939) suggested that the inhibitors of cholinesterase which are tertiary bases, such as eserine, give rise in the body to a lipoid-soluble base which penetrates the cells and acts as a convulsant, whereas the quaternary bases, such as prostigmine, give rise to a lipoid-insoluble substance which remains outside the cells and acts as a depressant. This explanation could account for their results and those of Kremer (1941) but not for those of most other authors. The same inhibitor of cholinesterase was found to have opposite actions at different levels in the central nervous system (Bonvallet and Minz, 1938; McKail, Obrador and Wilson, 1939); in the spinal cord the response varied with the reflex tested but there was no qualitative difference between eserine and prostigmine (Merlis and Lawson, 1939; McKail, Obrador and Wilson, 1939; Bülbring

and Burn, 1941). The anesthetic used and the mode of application seemed to influence the response. Most authors found a similarity of action between acetylcholine and eserine or prostigmine and attributed it to the cholinesterase inhibiting property of these substances. The following points strengthen this view:

(1) There is a relationship between the inhibition of cholinesterase activity of a substance *in vitro* and its central effects (Schweitzer and Wright, 1939) in spite of the fact that the substances examined are decomposed by cholinesterase at widely different rates (Easson and Stedman, 1936) so that certain discrepancies are to be expected. Particularly striking were the results obtained on removal of the urethane grouping. Eseroline and eseroline methiodide, derived from eserine and eserine methiodide respectively, had a feeble inhibiting action on cholinesterase *in vitro* and exerted only weak central actions. Removal of the urethane grouping from prostigmine considerably weakened but did not abolish its central inhibitory action on the knee jerk. Schweitzer and Wright conclude that central inhibition may be partly independent of inhibition of cholinesterase, whereas central excitation is wholly due to it. Mention may be made in this connection of the fact that eserine in large doses apparently has a direct paralysing action on the cells of the sympathetic ganglion (Feldberg and Vartiainen, 1934).

(2) As in the peripheral effects, the time course of the response to eserine indicates an indirect mechanism of action. There is a definite latency which varies according to the mode of administration and the dose used, but which is, so far as comparisons have been made, longer than that seen with acetylcholine under similar conditions. For instance, the onset of the response to intraventricular injections of a few gamma eserine is delayed for 12 minutes, that to acetylcholine for 30 seconds (Henderson and Wilson, 1936). With very strong concentrations of eserine (1 to 10 per cent) applied locally to the motor cortex there is a time lag of 6 to 7 seconds before motor activity sets in (Miller, Stavraky and Woonton, 1940). The effect on the knee jerk in cats started three minutes or more after the intravenous injection of a small dose of eserine and reached its maximum slowly (Schweitzer and Wright, 1937; 1938b); with larger doses the latency progressively shortened. Bülbring and Burn (1941) published tracings of the effects on reflexes of drugs injected into the spinal circulation: acetylcholine, nicotine and adrenaline acted almost immediately, i.e., within the first ten seconds, and the effect quickly reached a maximum. With eserine or prostigmine there was a latency of 20 to 30 seconds and the effect increased gradually during the next minute or two. Gesell and Hansen (1943) point out that the eserine hyperpnoea develops slowly and disappears slowly, reflecting a gradual accumulation and disappearance of acetylcholine. The effects of subcutaneously injected eserine and prostigmine on the *petit mal* activity in epileptics occurred after a latency of 20 minutes or longer (Williams and Russell, 1941). There was an even longer latency before the effects on the spinal cord developed in man after intrathecal injections of eserine or prostigmine (Kremer, 1941).

*Literature before 1934.* Most of the early workers, using more or less impure preparations, observed effects which were attributed to central depressant and excitatory effects. Fraser (1867) who discovered the pharmacological potency of physostigmine, attributed the respiratory failure produced by the active principle of the calabar bean to a central action of the drug. It also produced a slight evanescent rise in body temperature. Fraser described many experiments which show that eserine causes paralysis of the spinal cord in frogs, rabbits and dogs leading to complete loss of reflex function. No effects were observed when the drug was applied directly to the cerebral cortex of frogs, birds and mammals; when it was applied to the spinal cord peculiar twitches occurred in the limbs,

followed by paralysis. Strychnine convulsions were abolished or prevented. He believed "that no other drug so directly diminished reflex action" as physostigmine and recommended extracts of the calabar bean against epileptic fits and tetanus. Its use was also recommended by other authors against these and other conditions of increased reflex excitability, strychnine poisoning and chorea; for references, see Harnack and Witkowski (1876). These authors, on the other hand, showed that a subcutaneous injection of physostigmine into an epileptic idiot led to numerous fits and psychic disturbances and that fits closely resembling those of epilepsy may be produced in specially operated guinea pigs. Brown-Séquard (1860) had shown that a convulsive affection resembling epilepsy can be produced by certain lesions in the spinal cord; subcutaneous injection of physostigmine into such animals was followed by an enormous number of fits during the next hours.

According to Harnack and Witkowski pure physostigmine had a central depressant effect only; the preceding stage of excitation observed by numerous workers was attributed to impurities. Harnack and Witkowski themselves however, observed this initial excitation with pure physostigmine. Heubner (1905) found that physostigmine usually caused depression in frogs, but sometimes convulsions which with one preparation were produced as regularly as with strychnine. In mammals excitation preceded paralysis; epileptic fits were produced in dogs and could be suppressed by chloroform. Rothberger (1901) concluded that excitation was probably due to cortical stimulation and that physostigmine, in suitable doses, stimulated the respiratory centre. The depression observed in frogs affected first voluntary movements, then reflex movements and finally respiration. According to Harnack and Witkowski motor and sensory regions were affected in mammals; according to Roeber (1868) there was complete paralysis of the nerve cells for pain whereas those for touch and muscle sense remained unaffected by physostigmine. Strong central excitation was obtained with physostigmine by Langley and Kato (1914). In cats and rabbits twitching of the eyelids and nystagmus occurred occasionally, synchronous on both sides and, therefore, undoubtedly central in origin. Similar synchronous movements and other muscular contractions were observed in birds. The brief rise in arterial blood pressure and the secretion of sweat from the cat's pad observed after physostigmine were thought to be mainly central in origin. They concluded that small doses of physostigmine set up new nerve impulses but they left open the question whether these were in part or wholly due to increased irritability of central synapses. Stewart and Rogoff (1921) described a centrally produced secretion of adrenaline by eserine which, however may have been caused reflexly and not by a direct action of the eserine on the central nervous system.

*Literature after 1934. Cortex.* Miller (1937), using anesthetised cats, placed bits of blotting paper soaked in 1 or 10 per cent of eserine solution on the area of the motor cortex for the fore and hind leg. The muscles of the contralateral leg contracted, the leg became rigid and during the following minutes tremors and a gradually increasing clonus set in. Excision of the eserinised motor cortex promptly softened the affected leg. The response to eserine was stronger

than that to faradisation of the cortex, which sometimes consisted mainly of flexion while that to eserine consisted mainly of extension. Tremors and clonus were specific for eserine. Removal of the blotting paper caused the symptoms to subside after several minutes; afterwards the threshold for cortical faradisation was lowered and flexion or clonus sometimes continued as after discharges. Later Miller, Stavraky and Woonton (1940) found that local application of eserine to the motor cortex of cats and rabbits caused within a minute or two a progressive reduction in amplitude of the slow and large fast waves in the electroencephalogram: the small fast waves and the dot waves became more apparent. These changes being associated with the described motor phenomena represented cortical stimulation. The reduction in amplitude was attributed to asynchronous firing of large numbers of neurons, owing to stimulation of multitudes of synapses in widely different degrees. Sjöstrand (1937) had previosly stated that eserine applied to the cortex together with strychnine was more active than strychnine alone in evoking large diphasic waves from the cerebral cortex. Chatfield and Dempsey (1941) applied 1 per cent prostigmine to the cerebral cortex of cats and observed depression of electrical activity, sometimes localised to the area of application, sometimes spreading to remote areas; later, spontaneous bursts appeared. Williams and Russell (1941) injected small subcutaneous doses of eserine and prostigmine into epileptics; prostigmine usually increased, whereas eserine decreased, the *petit mal* activity recorded in the electroencephalogram. As *petit mal* activity may well be the result of depression of nervous activity, the results are not necessarily at variance with the theory of Schweitzer et al. concerning quaternary and tertiary anticholinesterases. There was no effect on the encephalogram of normal persons. The intrathecal injection of prostigmine and eserine in man sometimes caused headache and in one case disturbances of speech (Kremer, Pearson and Wright, 1937; Kremer, 1941). Unlike prostigmine, eserine produced striking sensory changes including facilitation of sensory transmission (Kremer, 1941).

The effects observed in the isolated cat's head perfused with diluted blood may be due to cortical stimulation: eserine added to the blood caused hyperexcitability of the existing reflexes or their reappearance if they had disappeared; spontaneous blinking at the rate of about 80 per minute and jerking movements of the head sometimes occurred; the effects disappeared after section of the nerves; at later stages of eserine poisoning, depression set in (Chute, Feldberg and Smyth, 1940). Increased reflex excitability of the head was also obtained after injection of eserine into the carotid artery of cats (McKail, Obrador and Wilson, 1941); at the same time the motor response of limb and face muscles to electrical stimulation of the cortex was depressed. There was some suggestion that eserine occasionally potentiated the cortical response to central vagus stimulation.

*Cerebellum.* In one decerebrate cat Miller (1937) placed tablets of eserine near the centre of the anterior lobe of the cerebellum: "almost at once the right foreleg was thrust forward, the left likewise but more slowly. The hindlegs relaxed promptly, then stiffened, later relaxed and stiffened alternatively."

*Brain stem, medulla.* In man the intraventricular injection of 10 μg. eserine, a

dose which was ineffective intravenously, produced repeated vomiting, slight sweating and slowing of the pulse (Henderson and Wilson, 1937). Kremer (1941) observed vomiting, sweating, pallor and drowsiness after intrathecal injection of both eserine and prostigmine; emptying of the bladder was abolished.

*Respiratory centre.* Both prostigmine and eserine, given intravenously to cats under chloralose anesthesia, depressed respiration; the prostigmine depression was sometimes preceded and followed by stimulation. Cutting the vagi and eliminating the chemo-receptors did not materially alter the response. But in decerebrate cats the more pronounced stimulating effect of prostigmine on respiration was mainly a response of the chemo-receptors. Depression was observed with several other quaternary anticholinesterases (Schweitzer and Wright, 1938; 1939). In anesthetised dogs with eliminated chemo-receptors eserine, injected into a vertebral artery, caused a slowly developing hyperpnoea, sometimes preceded by diminished ventilation; return to normal respiration required hours (Gesell and Hansen, 1943). The effect resembled that produced by a slow arterial infusion of acetylcholine. The authors emphasize the difference between inhibition and paralysis of the centre: when eserine had caused arrest of respiration by inhibition, stimulation of the sinus nerve produced a more powerful response than before eserine, but after excessive eserine poisoning the centre became paralysed and no longer responded to afferent impulses. The stimulating action of eserine and prostigmine on "the crossed phrenic phenomenon" is probably a central effect of these drugs, but a possible action on the chemo-receptors was not excluded (Seligman and Davis, 1941).

Dikshit (1934) found no change in the respiratory response to central vagus stimulation in cats, when eserine was given intravenously or intraventricularly, but Gesell, Hansen and Worzniak (1942) found in dogs that subthreshold doses potentiated the response to stimulation of the sinus or of the superior laryngeal nerve.

*Spinal cord. Spontaneous discharge.* Lefebre and Minz (1936), by applying eserine locally to the isolated spinal cord of frogs, observed a change in reflex excitability suddenly followed by a discharge of motor impulses. In both anesthetised and decerebrate cats after atropine the intravenous injection of large doses of eserine caused convulsions and a marked increase in muscular tone. The effects were abolished after nerve section and were due to an action of the spinal cord as they were seen equally well after its division in the midthoracic region. Smaller doses caused increased reflex excitability. Other inhibitors of cholinesterase were examined; tertiary amines acted like eserine but quaternary bases, like prostigmine, depressed the cord and were able to abolish temporarily eserine and strychnine convulsions (Schweitzer and Wright, 1937; Schweitzer, Stedman and Wright, 1939; Calma and Wright, 1944). In dogs Merlis and Lawson (1939) observed a spontaneous discharge confined to myotomes innervated from the cord segments perfused with eserine. Increase of muscle tone was obtained after intravenous injection of a large dose of eserine. Bülbring and Burn (1941) observed a spontaneous discharge in dogs after injecting eserine

or prostigmine into the separate cord circulation. Nicotine, which had no action of this kind when given alone, caused a spontaneous discharge after subthreshold doses of these drugs.

*Reflexes. Knee jerk.* Schweitzer and Wright (1937; 1938c) and Schweitzer, Stedman and Wright (1939), using cats anesthetised with chloralose, found that the intravenous injection of eserine and other tertiary anticholinesterases augmented the knee jerk whereas prostigmine and other quaternary anticholinesterases depressed it. The increased excitability to eserine was often preceded and sometimes followed by a phase of diminished reflex activity. When the knee jerk had been abolished by an overdose of the anesthetic or by ergotoxine or prostigmine it was restored and even enhanced by eserine. In most of their experiments atropine was given intravenously to avoid the circulatory effects of eserine. The same difference in the action of eserine and prostigmine was observed in man after intrathecal injection by Kremer (1941). In a case of spinal block where the reflex was absent it could be evoked after eserine. Merlis and Lawson (1939) worked on dogs usually with the cord transsected; they perfused the subarachnoid space or applied the eserine locally to the cord and found that the knee jerk was usually depressed. After intravenous injection of eserine the reflex was augmented in over 40 per cent of experiments when chloralose and in 10 per cent when barbital was used as the anesthetic. Bülbring and Burn (1941) observed depression and abolition of the reflex after eserine or prostigmine had been injected into the separately perfused cord circulation of dogs, in spite of the fact that the drugs caused spontaneous discharges. Eserine, however, exaggerated the rebound which sometimes followed the inhibition of the knee jerk caused by homolateral stimulation of the posterior tibial nerve. The effects of nerve stimulation resembled those of injections of acetylcholine both before and after eserine. *Flexor reflex.* In cats, augmentation of this reflex occurred after intravenous injection of eserine (McKail, Obrador and Wilson, 1941). In dogs the reflex was examined by Merlis and Lawson (1939) and by Bülbring and Burn (1941). After intraspinal injection, eserine, even in large doses, always augmented the reflex. After intravenous injection, Merlis and Lawson observed depression in 5 per cent of cases. Prostigmine had a much weaker stimulating action which was only obtained in the presence of adrenaline in the cord circulation. In man (Kremer, 1941) intrathecal injection of eserine increased, and that of prostigmine abolished flexor reflexes. *The crossed extensor reflex* was increased in dogs after eserine (Merlis and Lawson, 1939). Torda perfused spinal toads from the aorta with concentrated solutions of prostigmine, but eliminated peripheral effects. As with acetylcholine the crossed extensor reflex was depressed and was no longer inhibited by homolateral stimulation of the sciatic nerve, which now evoked a large response. The results resemble those obtained by Bülbring and Burn on the knee jerk.

*Other reflexes and spinal activity.* In frogs eserine diminished the height of the reflex twitch of the semitendinosus provoked by homolateral stimulation of the sciatic nerve, but augmented the after discharge in the electromyogram (Bonnet and Bremer, 1937a). McKail, Obrador and Wilson (1939) found that eserine

usually depressed the response to stimulation of the pyramidal tract, like that to stimulation of the cortex, but in one instance increased the pyramidal tract response. In man (Kremer, Pearson and Wright, 1937; Kremer, 1941) muscle tone and reflexes were depressed by prostigmine injected intrathecally and the voluntary muscle movements were impaired, but in patients with hemiplegia these improved on the affected side, probably due to abolition of spasticity. The impairment of micturition also might have been a spinal effect. In a case of spinal block, prostigmine injected below the level of the block abolished muscle tone, spasm and reflexes. The effects of eserine however were mainly excitatory. After an initial depression of the spinal reflexes they were augmented with increased power in the legs, although no muscle spasm could be seen or felt. In the case of spinal block which displayed complete flaccid paralysis of the legs and loss of reflexes, the latter were re-established after eserine.

3. THE EFFECTS OF ATROPINE ON THE CENTRAL ACTIONS OF ACETYLCHOLINE, AND ESERINE PROSTIGMINE. Most authors have found that atropine antagonises the stimulating as well as the depressant central actions of acetylcholine, eserine and prostigmine, although there is a number of contrary statements. For instance, Dikshit (1934) injected atropine intravenously or intraventricularly without observing any effect on the inhibitory action of acetylcholine applied to the respiratory centre; similarly Shuh, Wang and Lim (1936) found that atropine, injected intravenously, was without effect on the stimulating action of intraventricular injections of acetylcholine on blood pressure and respiration. Farber and Heymans (1936) could not abolish by means of atropine the spleen contraction of central origin produced by acetylcholine. Schweitzer and Wright, Schweitzer, Stedman and Wright and Calma and Wright performed most of their experiments with anticholinesterases on atropinised cats because they had found that atropine did not materially alter the result. Merlis and Lawson (1939) found that neither the depression of the knee jerk nor the enhancement of the flexor reflex caused by eserine was affected by atropine; Kremer (1941) found atropine ineffective on the responses in man to intrathecal injections of eserine and prostigmine. Similarly Brenner and Merritt (1942) found that intravenous injections of atropine had no influence on the responses produced by acetylcholine applied in strong concentrations to the cerebral cortex or injected intracisternally.

The following observations, however, provide evidence for an antagonistic effect of atropine: Langley and Kato as early as 1915 had stated that the irregular eserine contractions of the limbs, which were central in origin, did not occur after atropine. According to Schweitzer and Wright (1937) atropine usually decreased or abolished the paralysing effect of acetylcholine on the knee jerk; according to Calma and Wright (1944) it abolished the augmentor effect of acetylcholine on the muscle tone in decerebrate cats. Atropine abolished the changes observed by Bonvallet and Minz (1937; 1938) in reflex excitability produced by acetylcholine in spinal and mid brain animals as well as the increase in excitability of the linguo-maxillary reflex produced by acetylcholine or eserine in dogs and cats (1938b). Bülbring and Burn (1941) found that atropine

abolished both the discharge of motor impulses from the spinal cord and the changes in reflex excitability produced by acetylcholine, eserine or prostigmine. According to Schweitzer and Wright atropine diminished the depressant effect of acetylcholine on the respiratory centre; after atropine only large doses were effective. Gesell and Hansen (1943) found that atropine reversed each of the changes in breathing which had been set up by eserine or acetylcholine. Miller (1943) found that intravenous injection of atropine abolished the effects on deglutition, respiration and tongue muscle produced by local application of acetylcholine in low concentration to the floor of the fourth ventricle. Similarly atropine prevented or abolished the stimulating effects of intraventricular injections of acetylcholine or eserine in man (Henderson and Wilson, 1936). The changes produced by acetylcholine in the response of the cortex to electrical stimulation were abolished by atropine; but the changes produced by eserine in the response to cortical or pyramidal tract stimulation were not wholly antagonised. Once eserine had abolished the electrical response atropine could not restore it (McKail, Obrador and Wilson, 1939). According to Miller, Stavraky and Woonton (1940) atropine given intravenously or applied to the cortex prevented the changes in the electroencephalogram produced by the local application of acetylcholine or eserine, whereas Chatfield and Dempsey (1941) found that atropine, injected intravenously into cats, abolished the spikes, which occurred in the intervals between bursts, but none of the other many changes in cortical activity produced by local application of strong solutions of acetylcholine or prostigmine. The effects of eserine, prostigmine, carbaminoylcholine and acetylcholine on the *petit mal* activity of the cortex in epileptics were abolished by atropine (Williams and Russell, 1941; Williams, 1941).

*Central effects of atropine when given alone.* It is well known that atropine has stimulating effects on the medulla and higher centres and only ultimately causes central depression. There is at present no justification for interpreting the stimulating action of atropine as an abolition of central depressant actions of released acetylcholine. There is another well known action of atropine, its sedative effect on the rigidity and tremor of parkinsonism. It is tempting to regard this effect as a central atropine-acetylcholine antagonism, similar to that observed when both drugs are applied artificially to the central nervous system. In fact there are a few recent observations suggesting that atropine may abolish central activity. Schweitzer and Wright (1937), unlike Bülbring and Burn (1941), found depression of the knee jerk on intravenous injection of atropine in doses not exceeding those necessary to abolish parasympathetic nerve effects. Often the depression was pronounced and no recovery took place. Bonvallet and Minz (1938b) saw inhibition of the linguo-maxillary reflex on intravenous injections of atropine into dogs and cats and Gesell and Hansen (1941) observed appreciable diminution of breathing when small doses of atropine were injected into the vertebral artery of dogs with eliminated chemoreceptors and double vagotomy. McKail, Obrador and Wilson (1939) describe a definite effect of atropine on cortical activity: the motor response to electrical cortical stimulation was depressed by $CO_2$ and by central vagus stimulation; atropine abolished the

$CO_2$ depression and mildly antagonised that caused by central vagus stimulation. Brenner and Merritt (1942) found that atropine applied to the normal cortex produced changes in electrical activity very similar to those produced by atropine on the eserinised cortex.

Despite these few observations the discrepancy remains that whereas atropine usually abolished the central effects of acetylcholine, applied artificially or allowed to accumulate by the action of eserine or prostigmine, it has little effect on the spontaneous and reflex activity of the central nervous system. Such a discrepancy has been observed also for peripheral effects and has been explained (Dale and Gaddum, 1930) on the assumption that the acetylcholine may be released in immediate contact with, or even inside, the cell membrane and that atropine is unable in these circumstances to intervene with its antagonistic action. This problem has been dealt with in detail by Dale (1938). With regard to the central nervous system the discrepancy is in fact not so great as in the peripheral nervous system because the atropine-acetylcholine or eserine antagonism was not obtained by all authors and because some central activity is abolished by atropine.

Atropine is not the only drug which abolishes the peripheral actions of acetylcholine. Scopolamine which is closely related chemically to atropine has a similar effect. If scopolamine had been used as widely as atropine has been in elucidating the mechanism of action of peripheral cholinergic nerves the parallelism between the peripheral and central actions of acetylcholine and nervous activity would have been much closer. Scopolamine, however, has not been examined as an antagonist to the central actions of acetylcholine, eserine or prostigmine.

There are other drugs which ought to be re-examined in the light of the theory of acetylcholine as a central transmitter. The actions of adrenaline have been reviewed recently (Burn, 1945) in this journal from the point of view of the acetylcholine theory and therefore will not be dealt with in this article.

4. ACETYLCHOLINE CONTENT OF THE TISSUE OF THE CENTRAL NERVOUS SYSTEM. Chang and Gaddum (1933) were the first to demonstrate with reliable pharmacological tests the presence of acetylcholine in brain and to give quantitative figures. In the next two years Dikshit (1934), Plattner (1934) and Kwiatkowski (1935) showed the presence of acetylcholine and its uneven distribution in the central nervous system. According to Dikshit, the cerebral cortex contained a little, the cerebellum much less acetylcholine than the basal ganglia; Kwiatkowski found more in the thalamus region than in the cortex. Some of the acetylcholine values given by these early workers are probably incorrect because they used methods which did not extract all the acetylcholine or allowed loss or synthesis to occur during the procedure. This argument applies also to the values given by Corteggiani, Gautrelet, Kaswin and Mentzer (1936) and by Fegler, Kowarzyk and Lelusz-Lachowicz (1938). In order to extract all the acetylcholine without loss or new formation the tissue has to be extracted either with well acidified (HCl) saline and boiled or with trichloracetic acid. The mincing and grinding must be carried out in these solutions since, if carried out in saline or in eserinised

saline, loss or new formation of acetylcholine respectively may occur. The final neutral extract must be assayed not against pure acetylcholine solutions, as is usually done, but against acetylcholine solutions containing equivalent amounts of tissue extract, the acetylcholine content of which has been destroyed by short treatment with alkali; otherwise the values obtained may be too high, particularly when the acetylcholine content of the tissue is low.

Acetylcholine has been isolated and identified chemically from the optic ganglion of octopus (Bacq, 1935) and later from ox brain which had been minced and incubated in chloroform before extraction to increase the yield of acetylcholine by synthesis (Stedman and Stedman, 1937).

The acetylcholine content of different parts of the central nervous system compared with values obtained from the peripheral nervous system is given in table 1 which summarises roughly the results obtained by the following workers: Chang and Gaddum (1933); Plattner (1934); Barsoum (1935); Bacq (1935); Dikshit (1938); Loewi and Hellauer (1938); Haas (1939); MacIntosh (1939, 1941); Cortelli, Feldman and Gellhorn (1941); Feldberg (1943, 1945); Welsh (1943). MacIntosh who supplied most of the values obtained in dogs and cats summarises his results as follows: no part of the central nervous system is as rich in acetylcholine as some peripheral nerve trunks or sympathetic ganglia. Acetylcholine is found both in grey and white matter of the cerebral hemispheres, but in all regions the grey matter contains more than the white. In the spinal cord the acetylcholine is mainly confined to the grey matter. The afferent fibres in the dorsal columns and in the medulla and, what is more surprising, the pyramids contain little or no acetylcholine. It occurs in fairly high concentration in the basal ganglia and in the midbrain. This might suggest concentration of acetylcholine at central synapses or in relation to cell bodies. On the other hand there is a significant proportion of acetylcholine in parts of the corpus callosum, of the internal capsule and in the superficial layers of the pons, all of which contain few cell bodies, while the cerebellum which is rich in cells contains hardly any acetylcholine. Its presence in many parts of the central nervous system does not necessarily signify the presence there of cholinergic neurons, as MacIntosh points out, and its absence does not definitely exclude their absence, although it makes it more probable. MacIntosh assayed samples of ascending and descending tracts of the frozen spinal cord of horses in order to obtain more detailed knowledge, but the acetylcholine content was too low to give reliable information.

A low store of acetylcholine with a great ability to synthesize it may be a characteristic feature of a tissue which like the central nervous system exhibits continuous activity. When considering the theory of acetylcholine as central transmitter it is necessary to realize that the acetylcholine content of brain apparently decreases with the phylogenetically higher development of the species.

The acetylcholine content of individual brains of the same species varies greatly but not that between the right and left halves of the same brain. On the average large guinea pigs contain a higher concentration of acetylcholine in

their brains than small ones, suggesting an increase with age (Feldberg, 1945). This result is supported by experiments made by Welsh and Hyde (1944). They ground rat's brain in ice cold esernised saline solution for a given time thus bringing an unknown proportion of the tissue acetylcholine into solution. They used brains of new born, young and adult rats and found that the amounts of acetylcholine increased with age, but to speak of this acetylcholine as free acetylcholine of the brain is misleading. Results obtained by the same method suggest that the acetylcholine content of the different parts of brain changes with age. In new born rats it appeared to be lowest in the pallidum and highest in the medulla, in adult rats lowest in the cerebellum and highest in the brain stem. It is unfortunate that the authors made no determinations of the actual acetylcholine content of the tissues. Welsh and Hyde point out that there is a general agreement in the quantitative distribution of acetylcholine in the central nervous system of the growing rat and that of the developing chick as determined by Szepsenwol and Caretti (1942). In the early chick embryo the medulla contains much acetylcholine which diminishes with age; in the diencephalon and mesencephalon the acetylcholine increases from a medium amount in the early stages to a high level which is maintained in the adult bird; the cerebrum at first has very little acetylcholine, but this increases and then diminishes again; the cerebellum shows a medium amount of acetylcholine in the early stages which decreases in the older chick. According to Kuo (1939) acetylcholine appears in chick embryos before synapses are found and there is no relation between increase in acetylcholine and development of the reflexes or of the nervous system.

*The form in which acetylcholine is present in the nervous tissue.* If acetylcholine were not protected against cholinesterase it could be present in the tissues in traces only, if at all. To explain this protection it might be assumed that acetylcholine is present in a diffusible form inside the cell but spatially separated from the tissue cholinesterase. The cell would form a diffusion-tight compartment and the release of acetylcholine could be visualised as an increase in the permeability of the membrane; injury of the membrane would bring acetylcholine into solution and into contact with cholinesterase so that it would be destroyed at once. This conception, as pointed out by Beznak (1934), is certainly wrong. When nervous tissue is divided mechanically by grinding it in sand, mincing or even homogenising it, the cell debris which can be spun down by centrifugation or seen under the microscope retains acetylcholine. Some acetylcholine is freed in the process and brought into solution; the debris, however, by synthesis replaces its acetylcholine so that on extraction it yields its normal complement. The wrong interpretation of this observation led to the assumption of an inactive "precursor substance" or of two kinds of acetylcholine, "bound" and "free", present in the tissue. As cell debris does not dialyse it is not surprising that the so-called precursor substance was found to be non-dialysable. By boiling a suspension of finely divided tissue or treating it with acid the cell debris is destroyed and its acetylcholine brought into solution; consequently the activity of such extracts increases. This is the explanation of the results obtained by Corteggiani and her co-workers, as pointed out by Trethewie (1938).

Acetylcholine is bound in the tissue or cell granules to some cell constituents;

it is synthesized into this linkage and in this condition is relatively immune to cholinesterase. The cell constituents responsible for the linkage are thought to be the lipins as well as the proteins. Chang and Gaddum (1933) observed that alcoholic extracts were less active when tested for acetylcholine than trichloracetic acid extracts; but Barsoum (1935) found that the deficit could be recovered when the alcoholic extract was subsequently treated with trichloracetic acid. An alcoholic extract, first freed from alcohol and then shaken with ether to remove fats, contained a certain amount of insoluble material in suspension. Such extracts when kept overnight lost about half their activity which, however, could be recovered by treating the extract with trichloracetic acid. The disappearance of activity was apparently due to the formation of an inactive compound. No active substance was formed from lecithin when treated with trichloracetic acid. Similar results were obtained by Loewi and Hellauer (1938) using tissue of frog's central nervous system. Acid alcohol extracts were found to be only half as active as trichloracetic acid extracts; nevertheless all the acetylcholine of the tissue had been extracted, but half of it had been converted into a saline insoluble form from which it could be freed and made water soluble by trichloracetic acid. They found that acetylcholine, which is practically insoluble in ether, became very soluble in ether when it was added to an alcoholic extract of nervous tissue and that the acetylcholine of the tissue could be removed to about 80 per cent from an acid alcoholic extract by the addition of ether. The ether solution obtained in this way showed slight opalescence. Acetylcholine added to acid alcohol without nervous tissue did not become ether soluble (Loewi, Hagen, Kohn and Singer, 1937; Loewi and Hellauer, 1938). From these facts they concluded that acetylcholine is linked in the tissue to the lipoids; this linkage would not explain why acetylcholine cannot be extracted from the nervous tissue by ether without a preceding extraction with acid alcohol; therefore they assumed an additional linkage to another tissue constituent, probably to the proteins.

In whatever linkage the acetylcholine is held we have to assume a kind of dynamic equilibrium in order to explain the findings of an apparently fixed acetylcholine content of the tissue. The tissue is able to store a definite but limited amount of acetylcholine which may be determined by the number of available cell constituents to which the acetylcholine is linked (see also section 8). Nevertheless synthesis is proceeding continuously at a slow rate, the excess of acetylcholine being released and at once destroyed. There is no evidence that "free" acetylcholine exists or can exist in the tissue. Even the tissue acetylcholine may not be completely immune to cholinesterase, but synthesis, as long as it proceeds, compensates any loss that may occur through enzymatic hydrolysis and thus allows the acetylcholine to attain and maintain certain definite levels characteristic for each part of the nervous system, for each species and for the age of the animal. When synthesis, however, is impaired or lost, the acetylcholine disappears from the tissue, as is the case with peripheral nerves in the early stages of degeneration (Feldberg, 1943) or with brain tissue kept frozen for several days (Feldberg, 1945).

Some of the protection of the tissue acetylcholine against the cholinesterase

is apparently lost when brain tissue is perfused for several minutes with saline solution devoid of eserine; it was found that the acetylcholine content of the guinea-pig's brain decreased under such circumstances, but when eserine was present in the perfusion fluid it remained normal. Similarly the cell granules of brain pulp gradually lose part of their acetylcholine when kept for some time in saline solution without eserine; in presence of eserine, however, no apparent change in their acetylcholine content occurs (Feldberg, 1945). In view of these observations the findings of Cortell, Feldman and Gellhorn (1941) that an intravenous injection of eserine into rabbits increases the acetylcholine content of the brain needs confirmation.

It is possible that the lipins, apart from any rôle they may play in the linkage of acetylcholine, confer upon the tissue acetylcholine its relative immunity to cholinesterase. Such an assumption would explain the fact that cell debris of fresh brain pulp, which on suspension in saline solution retains its acetylcholine store for some time, no longer does so when ether is also present. In presence of ether eserine must be added in order that the particles may retain their acetylcholine or regain it when it has disappeared through the absence of eserine (Feldberg, 1945).

Brain tissue dried *in vacuo* and powdered is unable to build up a store of acetylcholine in its particles when it is suspended in eserinised saline solution, but it acquires this property when suspended in an eserinised saline-ether mixture (Feldberg, 1945).

5. Synthesis of acetylcholine. Synthesis of acetylcholine by tissues of the central nervous system was discovered independently by Quastel, Tennenbaum and Wheatley (1936) and by Stedman and Stedman (1937). It occurs also in tissue of the peripheral nervous system and is a property not only of the nerve cell but also of the nerve fibre. The only non-nervous tissue in which it has been observed with certainty is the human placenta (Feldberg, 1943,1945). Mention, however, may be made of the fact that bacteria (*Bacterium acetylcholini*) are able to form acetylcholine (Keil and Kritter, 1935; Keil and Weyrauch, 1937/38; Möller and Ferdinand, 1937/38; Habs, 1937/38; Möller, 1938).

The synthesis of acetylcholine has been studied under the following three conditions:

(1) when brain slices or pulp are incubated in a saline medium,

(2) when brain pulp or dried powdered brain is suspended in a mixture of saline and chloroform or saline and ether respectively

and (3) when homogenised brain or a saline extract of acetone dried and powdered brain is used as the material for synthesis.

In all these conditions it is necessary to add eserine or another inhibitor of cholinesterase to the medium in order to prevent the hydrolysis of the acetylcholine formed, although it should be pointed out that the saline extracts from acetone dried brain contain only mere traces of cholinesterase. Under the third condition the enzyme system responsible for the synthesis is in solution and the acetylcholine is synthesized as free acetylcholine. No release of ace-

tylcholine from tissue particles is possible and synthesis therefore can be examined without interference from this factor. Under the first two conditions the enzyme system is still attached to the particulate matter, although not necessarily to intact cells. The acetylcholine is synthesized within the cells or cell granules and before being brought into solution must be released from them. As mentioned above, the tissue particles maintain their normal store of acetylcholine, or rather have the tendency to replace it as long as synthesis is allowed to proceed, but they appear to be unable to build up a store of acetylcholine higher than their normal physiological complement. Under these conditions, therefore, synthesis is closely linked with and dependent upon the release of acetylcholine and it is not always easy to distinguish clearly between effects promoting release and formation respectively. When brain slices or pulp are incubated in a saline medium synthesis occurs under conditions where respiration apparently is a dominant factor; on the other hand when brain pulp or dried brain is suspended in a mixture of saline and chloroform or saline and ether respectively, or when dried brain is suspended in saline solution, synthesis occurs under conditions where respiration is more or less absent.

(1) *Brain slices or pulp in a saline medium* were used first by Quastel, Tennenbaum and Wheatley; the method has been worked out in more detail by Mann, Tennenbaum and Quastel (1938, 1939) and used with success by several workers (Trethewie, 1938; Sykowski, Fazekas and Himwich, 1939; Emmens, MacIntosh and Richter, 1942; Feldberg, 1945). To obtain with this method optimal conditions of synthesis incubation should be carried out at 37°C. under aerobic conditions and the medium should contain a low concentration of glucose and a high concentration of potassium; up to 50 μg. acetylcholine are formed per hour by the equivalent of 1 gram of fresh rat's brain.

(2) *Brain pulp or dried brain suspended in a mixture of saline and chloroform or saline and ether respectively.* These media were introduced by Stedman and Stedman (1937; 1939). Originally they incubated their samples at 37°C., but at this temperature their results could not be confirmed by Mann, Tennenbaum and Quastel. Later they worked at room temperature and Mann et al. showed that the yield of acetylcholine in these media is much greater at room than at body temperature. The effect of temperature has been examined further by Feldberg (1945) using dried brain. An increase in temperature from 20 to 37°C. accelerates the synthesis, but has the additional effect of inactivating the enzyme system more quickly, so that synthesis comes to an end. Therefore, when synthesis is studied for periods longer than a few minutes, the yield is much greater at 20°C. than at 37°C. Although the oxygen uptake of dried brain is extremely small it synthesizes acetylcholine, when shaken in an ether saline mixture, only in the presence of oxygen; under anaerobic conditions synthesis comes to an end after a short time; some initial synthesis occurs however also in anaerobic conditions. When dried brain is suspended in ether without saline no synthesis occurs; on the other hand when the volume of saline used is too great a condition is reached in which a water soluble substance necessary for the synthesis is diluted beyond a point sufficient for the synthesis to proceed. With the dried powder from the guinea-pig's cortex the formation of acetylcholine per hour was 72 μg. per gram powder, which corresponds to about 16 μg. per gram fresh weight. The effect of ether was also obtained when its vapour was bubbled through a suspension of brain pulp in diluted plasma. Mann, Tennenbaum and Quastel explained the accelerating effect of ether and chloroform by their ability to facilitate the release of acetylcholine, thus affecting synthesis indirectly, because they found that the store of acetylcholine held in the tissue decreased in the presence of ether. However, the tissue particles of dried brain powder are able to build up their store

of acetylcholine when suspended in an eserinised saline-ether mixture. This observation does not exclude the possibility that ether, by its lipin solvent action, facilitates release of acetylcholine, but synthesis itself is probably also affected directly.

(3) *Homogenised brain and saline extracts of acetone dried brain.* Nachmansohn and Machado (1943) found that homogenised brain synthesizes acetylcholine anaerobically in the presence of adenosinetriphosphate (ATP) and fluoride, the fluoride being necessary to protect the ATP against phosphatase contained in homogenised brain. This discovery attributes a completely new rôle to ATP. Their main results have been confirmed (Feldberg and Mann, 1944, 1945a). According to Nachmansohn and Machado homogenised rat's brain synthesized under optimal conditions, i.e., in the presence of choline and a high concentration of KCl, 35 to 100 μg. acetylcholine per gram; even higher values were obtained with frog's brain. The values may be somewhat too high as their method of assaying acetylcholine on the frog's rectus muscle does not take into account the fact that tissue extracts and subthreshold doses of choline sensitize the muscle to the action of acetylcholine. Feldberg and Mann found that homogenised brain incubated aerobically but under otherwise similar conditions, formed small amounts of acetylcholine only. Better results were obtained with saline extracts prepared from acetone dried and powdered brains. The acetone treatment did not apparently affect the enzyme system, which could be completely separated from the particulate matter of the brain by the saline extraction. Under anaerobic conditions and in the presence of ATP, fluoride and a high concentration of KCl, an extract from 1 gram powder formed in one hour up to 400 μg. acetylcholine; this corresponds to about 70 μg. per gram fresh weight and to about 1400 μg. per gram dry material contained in the saline extract. Under aerobic conditions half these amounts only were formed. The inhibitory action of iodoacetate, copper ions and iodine on the synthesis of acetylcholine led Nachmansohn and Machado to postulate the presence of active SH-groups in the enzyme. Feldberg and Mann suggested that their oxidation to inactive -SS-groups could explain the inhibitory effect of oxygen. It was shown that in the presence of SH-compounds, such as reduced glutathione or cysteine, which would prevent or reverse the oxidation of the SH-groups in the enzyme, the aerobic synthesis of acetylcholine was brought to the same level as, or even to a higher level than, that observed anaerobically. These SH-compounds had some activating effect also on the anaerobic synthesis which suggested that some SH-groups in the enzyme system might have undergone oxidation in the course of the preparation or extraction of the acetone powder. The corresponding -SS-compounds, oxidised glutathione and cystine, inhibited the synthesis of acetylcholine both under aerobic and anaerobic conditions. It thus appears that ATP and SH-compounds or similarly acting substances are essential for the aerobic synthesis of acetylcholine. Unlike the homogenised brain tissue, the extracts prepared from the acetone powder liberated ortho-phosphate from ATP only very slowly. No relation was found between the rate of synthesis of acetylcholine and the rate at which free phosphate was split off. The function of ATP appears to be specific; even closely related derivatives such as adenosinediphosphate (ADP) or inosinetriphosphate were found to be poor substitutes of ATP. It is too early to state precisely which part of the ATP molecule is involved in the formation of acetylcholine. The evidence available so far suggests that the most likely change is the conversion of ATP to ADP (Feldberg and Mann, 1945).

Recently Feldberg and Mann (1945b) showed that ATP is not an irreplaceable component of the synthesizing system. An equally large formation of acetylcholine could be obtained both aerobically and anaerobically if, instead of ATP, citrate and boiled saline extract prepared from acetone dried brain were added to the synthesizing medium. If all three activators, ATP, citrate and boiled juice were added together to the brain extract, the rate of formation of acetylcholineper hour rose to 1200 μg (rat) and 1800 μg (guinea-pig) per gram acetone powder, i.e. to over 4 and 6 mg acetylcholine respectively per gram dry material contained in the saline extract.

*Some factors which influence the synthesis. Oxygen, cyanide, azide.* The apparently antagonistic action of oxygen in the different conditions under which the synthesis of acetylcholine has been studied has been pointed out. Quastel, Tennenbaum and Wheatley (1936) assumed that it was closely linked with cell respiration. There is no doubt that when the synthesis is studied in brain slices or pulp, incubated in a saline medium, it is abolished under anaerobic conditions and greatly inhibited by KCN under aerobic conditions. KCN as well as azide however, has no effect on synthesis under conditions where cell respiration is absent, e.g., when dried powdered brain, homogenised brain or saline extracts of acetone brain powder are used (Nachmansohn and Machado, 1943; Feldberg, 1945; Feldberg and Mann, 1944, 1945). It is possible that the respiration in brain slices and pulp is associated with the mechanism of the release of acetylcholine and so affects the synthesis indirectly. The fact, however, that a synthesis of acetylcholine occurs without oxygen, i.e., under conditions where cell respiration is absent, does not prove that oxygen is not necessary for one of the various reactions involved in the synthesis as it occurs in the living tissue. The rôle of oxygen for the synthesis in this condition is all but clear. It may well be that the synthesis *in vivo* occurs in two stages, the first, anaerobic, being dependent on the presence of ATP, the second, aerobic, on the presence of oxygen. Such a possibility is not too remote when it is remembered that metabolic processes, such as for instance the carbohydrate metabolism in muscle, occur in two successive stages, anaerobic reactions being followed by aerobic reactions.

*Choline and acetate.* Stedman and Stedman (1937) considered choline as the precursor of acetylcholine, but they found that it did not influence the synthesis in brain pulp when added to their chloroform-saline medium. Brown and Feldberg (1937), in experiments on sympathetic ganglia, found, although not regularly, that choline exerted a strong accelerating effect on synthesis and such an effect was observed regularly by Mann, Tennenbaum and Quastel (1938) in their experiments with respiring brain tissue. A much stronger accelerating action by choline was observed by Nachmansohn and Machado (1943) on the anaerobic synthesis of acetylcholine in homogenised brain in the presence of ATP. They therefore termed the enzyme responsible for the synthesis "*choline acetylase.*" Their results with choline have been confirmed both for the anaerobic and for the aerobic synthesis in homogenised brain and in saline extracts of acetone dried brain. The substrate from which choline is derived is unknown. It may be present as free choline. On the other hand, it may be, as suggested by v. Muralt (1943), that phosphatides like lecithin or sphingomyelin yield choline provided that the phosphoryl part is taken up by phosphate acceptors such as aneurin or adenylic acid. These acceptors would be phosphorylated to cocarboxylase and ATP respectively. There is as yet no conclusive experimental evidence for this theory. Nothing is known about the origin of the acetate group. Nachmansohn, Cox, Coates and Machado (1943) suggest that acetylphosphate or phosphorylcholine may be intermediary products. Sodium acetate had no effect on the synthesis under whatever conditions it was studied (Stedman and

Stedman, 1937; Mann, Tennenbaum and Quastel 1938; Kahlson and MacIntosh, 1939; Nachmansohn and Machado, 1943; Feldberg and Mann, 1944; 1945a). Acetoacetate was suggested by Stedman and Stedman (1937; 1939) as an esterifying agent for choline, as it increased synthesis by brain pulp incubated in a saline-chloroform or saline-ether medium, but it had no effect of this kind on the synthesis by brain pulp incubated in a saline medium or on the synthesis in intact sympathetic ganglia. Acetaldehyde was also without effect (Mann, Tennenbaum and Quastel, 1939; Kahlson and MacIntosh, 1939). As pointed out already, in saline extracts from acetone dried brain, citric acid has a particularly strong activating influence on the formation of acetylcholine. Thus the possibility arises that citric acid also may act as the source of the acetyl groups.

*Glucose and other sugar derivatives.* Glucose may accelerate or inhibit synthesis according to the condition under which it is used. It stimulates and is in fact essential for the aerobic synthesis of acetylcholine in brain slices or pulp incubated in a saline medium and this effect is inhibited by d,l-glyceraldehyde (Mann, Tennenbaum and Quastel, 1938, 1939). The optimal concentration for the stimulating action of glucose lies far below the blood sugar level. It is thus not surprising that the effect is not evident when glucose is added to large quantities of brain tissue incubated in a small volume of saline, because the natural glucose, or substances derived from the tissue and acting like glucose, are not sufficiently diluted to create a deficiency which added glucose might relieve. The failure of Stedman and Stedman (1939) and of Sykowsky, Fazekas and Himwich (1939) to observe an increased rate of synthesis under conditions apparently similar to those used by Mann et al. can be explained on these lines (Mann, Tennenbaum and Quastel, 1939; Feldberg, 1945). According to Mann et al., mannose, pyruvate and lactate acted like glucose; galactose had a weak stimulating action and the effect of fructose was doubtful. Hexosediphosphate as well as $\alpha$-glycerophosphate did not accelerate the synthesis when brain pulp was incubated, but did so when brain slices were used. Glucose had only a slight stimulating effect on the synthesis in brain pulp suspended in an ether-saline medium and it exerted no stimulating action when dried brain or saline extracts obtained from acetone powder of brain were used. These differences may be explained on the assumption that the stimulating action of glucose is linked with that reaction in the synthesis of acetylcholine which is dependent upon the tissue respiration; the effect cannot be due solely to increased release of acetylcholine, because in the absence of glucose nervous tissue appears to be unable to replace the acetylcholine released by nervous impulses, as was shown by Kahlson and MacIntosh (1939) in their experiments on perfused sympathetic ganglia.

Concentrations of glucose as high or higher than those normally present in blood were found to inhibit synthesis in brain pulp incubated in a saline medium (Feldberg, 1945). It is possible that this effect is due to a direct action of glucose on the release of acetylcholine, and that the synthesis is influenced only indirectly. Glucose has no inhibitory action on synthesis in brain pulp suspended in an ether-saline medium or when dried powdered brain is used, but pyruvate,

according to Stedman and Stedman (1937, 1939) inhibited synthesis in brain pulp suspended in a chloroform-saline medium. Glucose, fructose (but not sucrose) and certain phosphohexoses were found, even in small concentrations, to inhibit strongly the anaerobic as well as the aerobic synthesis in saline extracts of acetone powder in the presence of ATP (Feldberg and Mann, 1944, 1945). The effect could be attributed to an enzymic esterification of the labile phosphate groups of ATP. There is as yet no evidence that this mechanism accounts also for the inhibiting action which large concentrations of glucose have on the formation of acetylcholine in brain pulp. If instead of ATP, boiled juice from brain and citrate are used, the saline extract from acetone dried brain synthesizes acetylcholine equally well in the presence and in the absence of glucose (Feldberg and Mann, 1945b).

*Potassium and calcium.* Potassium accelerates and calcium diminishes the formation of acetylcholine in respiring brain slices or pulp. The action of the ions was thought to be one on cell permeability, i.e., on release of acetylcholine and not on synthesis proper. Potassium in fact was found to decrease the acetylcholine content of the tissue slices, but this result needs confirmation. Rubidium and caesium acted like potassium (Mann, Tennenbaum and Quastel, 1938, 1939). When dried brain powder was used, potassium affected the synthesis of acetylcholine only in a saline suspension and not in an ether-saline medium, whereas the inhibitory effect of calcium occurred in both media. Potassium also had no effect on the acetylcholine store held in the tissue particles of dried brain suspended in ether-saline, whereas calcium prevented this store from being built up, a fact which suggested that calcium at least acted on the synthesis proper. The finding of Nachmansohn and Machado (1943) that potassium did not accelerate anaerobic synthesis in homogenised brain could not be confirmed by Feldberg and Mann (1944, 1945a). They found that potassium increases and calcium decreases both the anaerobic and the aerobic synthesis of acetylcholine in homogenised brain or in saline extracts of acetone dried brain in the presence of ATP, that is, under conditions where the factor of release of acetylcholine from tissue particles is excluded. A study of the action which Ca and K have on the metabolism of ATP revealed the fact that the Ca-ions inhibit the breakdown of ATP to inorganic phosphate whereas K-ions do not affect the dephosphorylation of ATP. Thus the antagonistic action of the two ions is not solely dependent on the manner in which they affect the dephosphorylation of ATP; there must be yet another process connected with the synthesis of acetylcholine in which the two ions are involved.

The release of acetylcholine from cholinergic nerve fibres is generally regarded as the result of a "mobilization of K-ions," which occurs during the passage of a nerve impulse; the release of acetylcholine is followed by its resynthesis, a process said to be independent of the passage of the impulse. There is no doubt that the nerve impulse releases acetylcholine because the tissue store of acetylcholine can be decreased by nerve impulses when synthesis is prevented, as seen in experiments by Kahlson and MacIntosh (1939) on perfused sympathetic ganglia.

The fact that K-ions are involved in the formation of acetylcholine, however, suggests that the "mobilisation of K-ions" also has a direct effect upon the resynthesis. When the synthesis of acetylcholine is studied in particulate matter of brain tissue potassium ions apparently accelerate the synthesis and facilitate the release of acetylcholine; calcium ions, on the other hand, inhibit both synthesis and release.

*Mg and $NH_4$ ions.* Mg-ions and large concentrations of $NH_4$-ions inhibit synthesis in respiring brain slices (Mann, Tennenbaum and Quastel, 1939) but have no action of this kind on synthesis in homogenised brain or in saline extracts of acetone dried brain (Nachmansohn and Machado, 1943; Feldberg and Mann, 1944, 1945a).

*Amino and other acids.* The anaerobic synthesis of acetylcholine by homogenised brain in the presence of ATP diminishes greatly after dialysis; some reactivation occurs with citric and glutamic acids and to a smaller degree with glutamine, alanine, methionine and succinic acid (Nachmansohn, John and Waelsch, 1943). In saline extracts from acetone dried brain citrate strongly activates the synthesis of acetylcholine. Citric acid can not be replaced by succinic, fumaric or tartaric acids, but malonic, glutamic, and aconitic acid show a slight activating effect. Malonic acid does not inhibit the action of citric acid (Feldberg and Mann, 1945a). In intact sympathetic ganglia or in respiring brain slices succinate and -ketoglutarate are without effect on the synthesis of acetylcholine (Kahlson and MacIntosh, 1939; Mann, Tennenbaum and Quastel, 1939).

*Inhibitors.* Cu-ions, iodine and iodoacetic acid inhibit synthesis (Nachmansohn and Machado, 1943; Feldberg, 1945; Feldberg and Mann, 1945a). The effect of iodoacetic acid was observed with brain pulp incubated in saline solution, with dried brain suspended in an ether-saline medium, in homogenised brain and in saline extracts of acetone dried brain, the effect of Cu-ions under the last two conditions and the effect of iodine in homogenised brain. The anaerobic and even more the aerobic synthesis in saline extracts of acetone dried brain in the presence of ATP was inhibited also by ascorbic acid and by hydroquinone (Feldberg and Mann, 1945c).

*Vitamin $B_1$, cocarboxylase.* Mann and Quastel (1940) found that brain slices from vitamin $B_1$-deficient pigeons, incubated in a saline medium containing glucose, pyruvate and a high concentration of KCl, formed less acetylcholine than brain slices from normal pigeons incubated similarly. The synthesis in the brains from the vitamin-deficient pigeons could be restored to normal by the addition of cocarboxylase to the medium. Cocarboxylase had no effect on the synthesis in brain slices of normal pigeons. According to Torda and Wolff (1944), however, both cocarboxylase and vitamin $B_1$ in low concentrations slightly accelerate and in high concentrations inhibit, aerobic synthesis of acetylcholine in a saline suspension of homogenised frog's brain. On the other hand, neither cocarboxylase nor aneurin are able to replace ATP in the synthesis of acetylcholine in the saline extract of acetone dried brains. In fact, vitamin $B_1$ was

found to inhibit the anaerobic synthesis in the presence of ATP (Feldberg and Mann, 1945a).

*Adrenaline* increases the aerobic synthesis obtained in a saline suspension of homogenised frog's brain (Torda and Wolff, 1944).

*Quinine* according to Sykowsky, Fazekas and Himwich (1939) increases the rate of synthesis in respiring rat's brain slices.

*Stability of the enzyme.* Synthesis is greatly diminished or lost when intact brain, brain pulp or homogenised brain is kept frozen for several days (Nachmansohn and Machado, 1943; Feldberg, 1945). Brain dried in the desiccator or acetone dried brain, however, retain their ability to synthesize acetylcholine when kept in the cold (Feldberg, 1945; Feldberg and Mann, 1945).

*Synthesis of acetylcholine in different parts of the central nervous system and in different species.* Some caution is necessary when comparing the synthesis in different tissues. For instance, rat's brain synthesizes large amounts of acetylcholine when slices are incubated in a saline medium or when the homogenised brain or saline extracts of the acetone dried brain are incubated in the presence of ATP, but only small amounts are formed when dried powdered brain is suspended in an ether-saline medium. The evidence so far available suggests that synthesis does not increase with the phylogenetic development; on the contrary the highest rate of synthesis so far observed has been with frog's brain (Nachmansohn and Machado, 1943) and cat's, dog's and monkey's brain were found to be less active than rat's and guinea-pig's brain. The activity in different parts of the central nervous system decreases in the following order: cerebral cortex, brain stem, medulla, spinal cord, cerebellum (Feldberg, 1945). As the cerebellum is relatively rich in cells, synthesis is not simply related to the density of cells in the tissue. Although no data are available on the synthesis in various finer structures of the central nervous tissue comparable with those on the acetylcholine content, there appears to be a relation between the two. Those parts which are relatively rich in acetylcholine synthesize relatively large amounts of acetylcholine. The statement of Sykowski, Fazekas and Himwich (1939) that brains of new born rats synthesize acetylcholine at a greater rate than those of adult rats, which appears to contradict this conclusion, needs confirmation. The acetylcholine content of motor roots is much higher than that of any part of the central nervous system, but the ability of brain tissue of rats and guinea pigs to synthesize acetylcholine appears to be as high, if not higher, than that of motor roots (Feldberg, 1943; Feldberg and Mann, 1945c). Too few experiments have yet been carried out on the synthesis of acetylcholine in peripheral nervous tissue to allow a comparison with the synthesis in central nervous tissue. There is no strict parallelism between the choline esterase content of nervous tissue and its ability to synthesize acetylcholine; whereas there is a close relation in some tissues there is gross discrepancy in others. At present, experiments are lacking in which the same author has compared, in various nervous tissues, the acetylcholine content, the cholinesterase content and the ability to synthesize acetylcholine under different conditions.

6. RELEASE OF ACETYLCHOLINE. Only results obtained on intact tissue will be considered here; those obtained on brain slices, pulp or powder have been dealt with in the previous section. The evidence available so far suggests that stimuli which are effective in slices, pulp or powder are also effective on intact nervous tissue.

*Spontaneous release.* The central nervous system exhibits continuous activity and on the hypothesis of central synaptic transmission by acetylcholine, its continuous release in the absence of any external stimuli is to be expected. In fact, acetylcholine appears in the cerebrospinal fluid of dogs and cats after intravenous injections of eserine or when the ventricular system is perfused with eserinised solutions (Feldberg and Schriever, 1936; Chang, Hsieh, Li and Lim, 1938; Adam, McKail, Obrador and Wilson, 1938). The concentration of acetylcholine in the C.S.F. rose with the dose of eserine injected intravenously. This may be due to the fact that a more complete inhibition of the cholinesterase activity leads to a greater accumulation of acetylcholine and consequently to increased activity of the central nervous system. Chang, Chia, Hsu and Lim (1937) detected acetylcholine in the venous blood from the isolated dog's head vivi-perfused by a second eseinised dog. Chute, Feldberg and Smyth (1940) observed the output of small amounts of acetylcholine when they perfused the almost completely isolated cat's brain with 50 per cent defibrinated eserinised blood, and Bülbring and Burn (1941) made a similar observation when perfusing the spinal cord of dogs. The fact that Altenburger (1937) was unable to detect acetylcholine in human C.S.F. is probably to be explained by inability to inject into patients sufficiently large doses of eserine.

*Potassium* causes temporarily an increase in the small spontaneous output of acetylcholine from the perfused cat's brain (Chute et al., 1940).

*Adrenaline* causes the appearance of acetylcholine or an increase of its concentration in the cerebrospinal fluid of eserinised dogs (Feldberg and Schriever, 1936; Chang et al., 1938). The effect also occurred but with some delay when the pressor effect of adrenaline was prevented by the use of a compensator. In the experiments of Adam et al. (1938) adrenaline was ineffective. Adrenaline probably does not release the acetylcholine directly, a possibility discussed by Bonvallet and Minz, (1938) but lowers the threshold for the transmission of impulses within the central nervous system. This will result in an increased spread of a given sensory impulse and thus to increased release of acetylcholine (Bülbring and Burn, 1941).

*Pituitrin.* According to Adam et al. (1938) very large concentrations of pituitrin in the fluid used for perfusing the ventricular system double the acetylcholine concentration of the outflowing fluid. Pituitrin given intravenously did not have this effect.

*Asphyxia* causes the appearance of acetylcholine, or its increase, in the cerebrospinal fluid of eserinised dogs. The effect is obtained after removal of the suprarenal glands and is independent of the rise in arterial blood pressure (Feldberg and Schriever, 1936). Adam et al. (1938) found no such effect in dogs on breathing a mixture of 7 per cent carbon dioxide in air.

*Direct stimulation.* Strong faradic stimulation of the isolated cord of rabbits (Minz, 1936) and of the brain-cord preparation of toads (Li, 1938) caused the release of acetylcholine into the surrounding fluid. According to Li a constant current passing through the braincord preparation did not have this effect. The acetylcholine-like activity which, according to Blinova and Lobova (1936), appears sometimes in the cerebrospinal fluid and in the venous blood collected from the sinus after stimulation of the cerebral cortex cannot be due to acetylcholine because the dogs used for these experiments received no eserine. Adam et al. (1938) when perfusing the ventricular system with eserinised Locke solution, obtained no increase in the output of acetylcholine by stimulation of the motor or cerebellar cortex or of the spinal cord, but after stimulation of the hypothalamus the acetylcholine concentration in the perfusate rose to about 100 per cent in half the experiments. This may be associated with the release of acetylcholine from nervous connections to the posterior pituitary (see section 8).

*Stimulation of afferent nerves.* During central vagus stimulation Dikshit (1934) sometimes observed the appearance of acetylcholine in the cerebrospinal fluid of cats and Bykow (1935) and Benetato and Munteanu (1936) in the blood leaving the medulla. Feldberg and Schriever (1936) were unable to confirm the results of Dikshit in dogs. Negative results were obtained also by Adam et al. (1938) in their perfusion experiments on the ventricular system, when they stimulated the vagus centrally or other afferent nerves. According, however, to Chang and his co-workers (Chang, Chia, Hsu and Lim, 1937, 1938; Chang, Lim and Lu, 1938) stimulation of the central end of the vagus or of the sinus nerve leads to the appearance of acetylcholine in the venous blood from the isolated dog's and cat's head, vivi-perfused by a second animal. The effect is not wholly due to the release of acetylcholine from nervous connections with the posterior pituitary, as a delayed effect occurs also on hypophysectomised animals. In later experiments Chang, Hsieh, Li and Lim (1938) succeeded in demonstrating the appearance of acetylcholine, or its increase, in the cerebrospinal fluid after central vagus stimulation when eserine had been given not only intravenously, as in the experiments of Feldberg and Schriever, but also intraspinally. There is thus good evidence that central vagus stimulation leads to the release of acetylcholine. Bülbring and Burn (1941) found that, when they perfused the lower half of the spinal cord with eserinised saline solution instead of blood, reflex activity persisted for about 8 minutes. During this period stimulation of the central end of the cut sciatic nerve caused the appearance of acetylcholine (1 in 50 millions) in the venous effluent.

7. ENZYMATIC HYDROLYSIS OF ACETYLCHOLINE. Although it is possible that part of the released acetylcholine may be reconverted into its inactive form in the tissues, most of it is probably destroyed at once by cholinesterase. This conclusion is based not only on the presence of cholinesterase in the central nervous tissue but also on the direct evidence that when cholinesterase is inhibited by eserine or other anticholinesterases, and only then, does acetylcholine appear in the cerebrospinal fluid or in the venous blood, and that the accumulation of

acetylcholine under this condition produces the central actions expected from the pharmacological activity of eserine.

Dale, as early as 1914, postulated the presence of an enzyme in blood able to hydrolyse acetylcholine and responsible for the evanescent character of its response after intravenous injection. Loewi and Navratil (1926) showed that tissue extracts destroyed acetylcholine and stressed the enzymatic nature of the process. Galehr and Plattner (1927) demonstrated the quick destruction of acetylcholine by blood but thought that no enzymatic process was involved in the reaction. Its enzymatic nature, however, was proved by Engelhart and Loewi (1930) and by Matthes (1930). Stedman, and Stedman and Easson (1932) demonstrated the specificity of the enzyme responsible for the destruction of acetylcholine in blood and named the enzyme cholinesterase. Further proof for the specificity was given by Easson and Stedman (1937). The Stedmans and their co-workers, however, pointed out that blood cholinesterase behaved somewhat differently in different species. The possibility of the existence of different cholinesterases has been considered subsequently by different authors (Hall and Lucas, 1937; Alles and Hawes, 1940; Richter and Croft, 1942). Mendel and Rudney (1942) maintain that the cholinesterase present in serum and certain tissues is non-specific and they call it pseudocholinesterase. De Laubenfels (1943) and Alles and Hawes (1944) think it inadvisable to give this name to the serum enzyme. A different cholinesterase, acting exclusively on choline esters, is present according to Mendel and Rudney in brain tissue and in the red blood cells of some animals and is particularly effective at low concentrations of acetylcholine; high concentrations of acetylcholine inhibit its activity. Some caution therefore is necessary when results obtained with serum cholinesterase are applied to brain cholinesterase.

Stedman and Stedman (1935) were the first to show the presence of relatively large amounts of cholinesterase in brain tissue and its absence in cerebrospinal fluid. According to Altenburger (1937) the destruction of acetylcholine in human liquor proceeds about 250 times more slowly than in blood. The Stedmans found that the concentration of cholinesterase in the basal ganglia of cats is about twice that in the cortex. In table 2 the distribution of cholinesterase in the central and in the peripheral nervous system has been tabulated from the papers of Nachmansohn, 1937, 1938a, b; Martini and Torda, 1938; Nachmansohn, 1939a; Couteaux and Nachmansohn, 1940; Nachmansohn and Meyerhof, 1941; Nachmansohn and Hoff, 1944. The following conclusions may be drawn:

(1) The cholinesterase concentration is greater in grey than in white matter. But not every region rich in cells yields a high concentration.

(2) There is no increase in the cholinesterase concentration with the phylogenetic order of the species. In fact the reverse is true. The highest value was obtained with the head ganglion of the squid and some of the lowest values were obtained with human cortex.

(3) A comparison with the peripheral nervous system shows that some regions of the C.N.S., although rich in cells, do not contain a greater concentration of cholinesterase than peripheral nerves and that sympathetic ganglia of cats have a higher concentration than nearly all parts of the central nervous system of mammals.

TABLE 1

*Acetylcholine content of nervous tissue*

| ANIMAL | TISSUE | μG. ACETYLCHOLINE PER GRAM FRESH WEIGHT |
|---|---|---|
| | Central nervous system | |
| Ox | Optic nerve and chiasma | 0 |
| Man | Cortex white matter; medulla; cerebellum | 0.1–0.3 |
| Cat | Cerebellum; optic nerve | |
| Man | Corpus striatum; crux cerebri; spinal cord | 0.2–0.6 |
| Dog | Cortex white matter; medulla; pons; cerebellum | |
| Cat | Occipital lobe white matter; dorsal columns; pyramids | |
| Water buffalo | Cortex white matter; medulla; pons; corpus striatum; cerebellum | |
| Dog, cat | White matter | 0.2–1 |
| Man | Thalamus | 0.6–1 |
| Dog | Spinal cord; optic chiasma | |
| Water buffalo | Cortex; thalamus | |
| Dog | Whole brain | 0.5–2 |
| Rabbit | Whole brain; cortex | |
| Cat | Internal capsule | 0.2–3.3 |
| Dog | Corpus striatum; crux cerebri; thalamus; spinal cord | 1–2.7 |
| Cat | Medulla; pons; midbrain; olf. bulb; hypothalamus | |
| Rabbit | Cortex | |
| Man | Basal ganglia | 1.5–3 |
| Cat | Corpus striatum | |
| Rat, mouse | Whole brain | |
| Pigeon | Whole brain | |
| Cat | Cortex; corpus callosum | 0.9–4.5 |
| Cat | Thalamus; whole brain | 1.5–4.5 |
| Dog | Grey matter | |
| Cat | Region of III. nucleus; pons, superficial medial | 2.5–5 |
| Rabbit | Brain stem | |
| Guinea pig | Whole brain | |
| Frog | Spinal cord | |
| Ox | Retina | 5–6 |

TABLE 1—*Concluded*

| ANIMAL | TISSUE | μG. ACETYLCHOLINE PER GRAM FRESH WEIGHT |
|---|---|---|
| | Central nervous system—*Concluded* | |
| Cat | Basal ganglia | 7 |
| Frog | Whole brain | 5–10 |
| Octopus | Optic ganglion | 77 |
| | Peripheral nervous system | |
| Dog, cat | Posterior roots | 0–0.25 |
| Ox | Postganglionic sympathetic fibres | 0.6–1.5 |
| Cat | Somatic mixed nerves | 2.5–7 |
| Cat | Vagus; nodose ganglion | 6–9 |
| Dog | Ventral lumbar roots | 9–10 |
| Dog | Sympathetic cholinergic nerves | 6–14 |
| Cat | Somatic nerves, mainly motor | 6–16 |
| Cat | Ciliary ganglion | 12 |
| Sheep | Sympathetic ganglia | 8–19 |
| Cat | Anterior roots | 12–22 |
| Sheep | Sympathetic cholinergic nerves | 12–23 |
| Cat | Sympathetic ganglia | 10–44 |
| Cat | Sympathetic cholinergic nerves | 18–40 |

(4) A comparison with the distribution of acetylcholine in central nervous tissue, as shown in table 1, does not yield a great deal of information owing to the absence of sufficient parallel estimations of the two components. Some parts rich in cholinesterase are relatively rich also in acetylcholine. The two values agree fairly well with regard to white and grey matter, to species differences, to thalamus and basal ganglia and to sympathetic ganglia. On the other hand, the cerebellum which is relatively rich in cholinesterase contains very little acetylcholine and the motor roots show a discrepancy in the opposite direction. Hellauer (1939) used a different method of determining the cholinesterase concentration in nervous tissue and found good agreement between acetylcholine and cholinesterase content in rabbit's spinal cord and frog's C.N.S. but not in the peripheral nervous system.

When evaluating the figures given in table 2 some caution is necessary. According to Hellauer (1939) the distribution of cholinesterase differs from that

TABLE 2

*Cholinesterase activity of different parts of the nervous system*

Expressed in milligram acetylcholine hydrolysed by 100 mgm. finely divided tissue in 1 hour

| | | | |
|---|---|---|---|
| **Central nervous system** | | | |
| White matter, brain | (ox, dog) | | 0.25–0.34 |
| Spinal cord, cervical bulb | (rat) | | 0.24–0.43 |
| Spinal cord, lumbar bulb | (rat) | | 0.33–0.48 |
| White matter, spinal cord | (cat) | | 0.6 |
| Cortex | (man) | | 1.2 |
| Optic nerve | (dog) | | 1.6 |
| Cortex | (dog, ox) | | 2–3 |
| C.N.S. | (electric fish) | | 1–4 |
| Thalamus | (man) | | 2.7 |
| Thalamus | (ox, dog) | | 2–5 |
| Olf. bulb | (rabbit) | | 4 |
| Post. quadrig. bodies | (ox) | | 4 |
| Spinal cord | (ox, rat) | | 5 |
| Brain | (rat) | | 7 |
| Spinal cord | (man, dog) | | 6–9 |
| Cerebellum | (rabbit) | | 9 |
| Spinal cord | (rabbit) | | 10 |
| Brain | (rabbit) | | 11 |
| Ant. quadrig. bodies | (ox) | | 10–12 |
| Post. quadrig. bodies | (rabbit) | | 12.5 |
| Retina | (dog, ox) | | 13–20 |
| Spinal cord | (cat) | | 11–25 |
| Ant. quadrig. bodies | (rabbit) | | 25 |
| Brain | (chicken) | | 25 |
| Caudate nucleus | (ox, dog) | | 23–57 |
| Putamen | (man) | | 46 |
| Lentiform | (ox) | | 67–72 |
| Head ganglion | (squid) | | 260–465 |
| **Peripheral nervous system** | | | |
| Post. root | (dog) | | 1.4 |
| Sciatic nerve | (dog) | | 1 |
| Ant. roots | (dog) | | 2.9 |
| Spinal gangl. | (dog) | | 3 |
| Sympath. gangl. | (dog) | | 11–19 |
| Sympath. gangl. | (cat) | (N. 1938, b) | 18–21 |
| Sympath. gangl. | (cat) | C. & N. 1940 | 40–60 |
| Denerv. sympath. gangl. | (cat) | C. & N. 1940 | 20–25 |

shown in table 2. He found a much greater difference in the cholinesterase concentration of posterior and anterior roots; the values for the latter (dog) were

in fact about 60 per cent higher than those for rabbit's spinal cord. In addition he determined the cholinesterase content in the central nervous system of frogs and found it to be about 10 times higher than that of rat's spinal cord and about 6 times higher than that of dog's anterior roots, so that it would assume either the value of 50 or of 18 in table 2. Even in the hands of the same author different values are obtained for the same tissue. The value for the sympathetic ganglion varied more than 100 per cent; in 1938 Nachmansohn found it to be lower than after denervation in 1940 (see table 2).

On the other hand there is a definite relation between cholinesterase concentration and functional development of the different parts of the central system. In brains of chick embryos, which are fairly well developed at hatching, the concentration of cholinesterase rises during the last four days before hatching. In rabbits and rats the rise occurs during the first three weeks after birth when the brain develops (Nachmansohn, 1938a, b). In the sheep's foetus the cholinesterase is already high in the early stages of gestation in the spinal cord but rises in the brain, the functional development of which occurs later, in the last weeks of gestation (Nachmansohn, 1940). Similarly Youngstrom (1941) found a good relation between the order of morphological development of the different parts of the central nervous system of the human foetus and the increase in cholinesterase concentration.

Martini and Torda (1938) found in rats that the cholinesterase activity of the spinal cord diminished two days after transverse cord section in the parts distal to the section, but returned nearly to normal within 2 to 3 weeks with the return of reflex activity. After cutting and degeneration of the dorsal roots Nachmansohn and Hoff (1944) found in cats a slight decrease in the cholinesterase concentration of the posterior as well as of the anterior horn of the corresponding levels of the spinal cord. The change was small compared with the great individual variations.

8. CHANGES IN THE ACETYLCHOLINE METABOLISM DURING SOME CONDITIONS OF INCREASED AND DECREASED CENTRAL ACTIVITY. Experiments have been carried out in order to discover whether, under acute changes of central activity, changes occur in the acetylcholine content or in the activity of the cholinesterase or of the synthesizing enzyme of the brain tissue. From the results described in section 6 it would appear that nervous tissue is unable to undergo acute changes in its acetylcholine content, but some such changes have been reported. A striking opportunity for attributing the effects of convulsive drugs to inhibition of cholinesterase activity was given by eserine. In this section conditions not yet reviewed in detail will be discussed.

*Convulsions by strychnine, metrazol or tetanus toxin.* The acetylcholine content of the central nervous system of frogs was found to be increased after prolonged strychnine covulsions lasting for several hours (Loewi, 1937; Cortell, Feldman and Gelhorn, 1941). In the experiments of Loewi the content doubled. But no such changes were observed by Cortell et al. in the brains of eserinised rabbits killed after a 5 minutes period of convulsions by strychnine or metrazol. Ac-

cording to Fegler, Kowarzyk and Lelusz-Lachowicz (1938) a few minutes of strychnine convulsions decrease and of tetanus convulsions increase the acetylcholine content of the central nervous system of rabbits. Their method of extraction, however, does not determine the acetylcholine content of the tissue and the meaning of the results is obscure. Large concentrations of strychnine were found to inhibit cholinesterase activity (Ammon, 1934; Nachmansohn, 1938b). According to the *in vitro* experiments of Nachmansohn, strychnine concentrations of 1 in 25,000 and of 1 in 7,000 inhibit about 20 and 60 per cent respectively. The concentration of strychnine in the grey matter of the spinal cord is several times lower, about 2–6 μg./g. (Veit, 1935), so that it is doubtful if in this condition strychnine exerts any inhibitory action on the cholinesterase. In fact no changes were found by Pighini (1939) in cholinesterase activity of brains of dogs and rabbits during strychnine or tetanus convulsions and by Cortell et al. in brains and spinal cord of frogs after 4 hours of strychnine convulsions, despite the increased acetylcholine content of the tissue.

*Insulin convulsions.* No change has been observed in the acetylcholine content and cholinesterase activity of brain tissue of mice, rats or rabbits during hypoglycemic shock (MacIntosh, 1939; Mann, Tennenbaum and Quastel, 1939; Cortell, Feldman and Gelhorn, 1941). Welsh (1943) found that mincing rat's cortex in ice cold eserinised saline solution brought less acetylcholine into solution when the cortex was from a rat killed during hypoglycemic shock. His claim, however, to have demonstrated that insulin hypoglycemia causes a decrease in the level of acetylcholine in the central nervous system is not borne out by these results. The ability of minced brain incubated in a saline medium to synthesize acetylcholine was found to be the same when the brains were from normal or insulin convulsed rats (Mann, Tennenbaum and Quastel, 1939). A new suggestion has been made by Feldberg (1945) to explain the insulin convulsions, based on the observation (see section 5) that glucose, in concentrations present in blood, inhibits the synthesis or the release of acetylcholine in minced brain. According to this view the blood glucose would normally exert a restraining effect on the continuous release or synthesis of acetylcholine in the central nervous system; lowering the blood sugar would remove the glucose "brake" and allow the synthesis and release of acetylcholine to proceed at an abnormal rate. This explanation would account for the failure of previous attempts to correlate the convulsions with acetylcholine. The inhibition by glucose may be due to esterification and thus to inactivation of ATP, which appears to be involved in the synthesis of acetylcholine, or to an effect on the mechanism of its release. The hunger contractions associated with a lowering of blood sugar might be interpreted by a similar mechanism partly in the periphery, partly on the acetylcholine metabolism at the vagal centre.

*Asphyxia, $CO_2$.* The results of experiments on the release of acetylcholine by asphyxia and increased $CO_2$ pressure of the alveolar air have been given on page 624. Increased $CO_2$ pressure was found to have little or no effect on the knee jerk (Schweitzer and Wright, 1937) or on the flexor reflex but depressed the

response to electrical stimulation of the cortex like acetylcholine (McKail, Obrador and Wilson 1939). This effect was readily suppressed by atropine and sometimes potentiated by eserine which is "strong presumptive evidence of the release of acetylcholine in the central nervous system." Since the spinal reflexes were relatively insensitive to $CO_2$ the action was probably mainly on the cortex. The release of acetylcholine could be effected in one of several ways. $CO_2$ might increase the sensitivity of the nerve cells to acetylcholine and thus increase the spread of sensory impulses by analogy with the interpretation of the similar action of adrenaline. $CO_2$ might facilitate the release or accelerate the synthesis of acetylcholine. Ether (even as vapour) and chloroform increase the synthesis of acetylcholine in brain pulp *in vitro* and consequently it is possible that some of the excitatory effects of the induction stage of chloroform or ether anesthesia may be accounted for in this way.

*Anoxia.* Cortell, Feldman and Gelhorn (1941) found no change in acetylcholine and cholinesterase content of rabbit's brain when the animal had been subjected to low oxygen pressure. According to Welsh (1943), mincing rat's cerebral cortex in ice cold eserinised saline solution brings into solution less acetylcholine than normal when the rat has previously been subjected to low oxygen pressure. His claim that anoxia decreases the acetylcholine level of brain is not borne out by his experiments.

*Heat coma.* Keeping frogs for 15 minutes at 37° to 39°C., which produces a comatose condition, decreases the acetylcholine content and the cholinesterase activity of the central nervous system (Cortell, Feldman and Gelhorn, 1941).

*Vitamin $B_1$* deficiency caused no changes in the acetylcholine content of the cerebral hemispheres or cerebellum of pigeons or the brain of rats (MacIntosh, 1939). Mention has been made of the observation that the synthesis of acetylcholine is impaired in this condition and that cocarboxylase has a restorative effect.

*Release of posterior pituitary hormone.* There is some evidence that the nerve fibres responsible for the secretion of the posterior pituitary hormone are cholinergic. Mention has been made of a relatively high acetylcholine content of the hypothalamic region (table 1) and the release of acetylcholine into the cerebrospinal fluid on electrical stimulation of this region. In addition acetylcholine was found to cause secretion of posterior pituitary hormone (Pickford, 1938, 1939). These experiments were initiated by the observation of Chang and his co-workers that a reflex secretion of posterior pituitary hormone occurred on central vagus stimulation and was associated with a release of acetylcholine into the blood stream. In hypophysectomised animals this release of acetylcholine was delayed, indicating that in normal animals it was partly associated with the posterior pituitary (Chang, Chia, Hsu and Lim, 1937, 1938; Chang, Lim and Lü, 1938).

*Barbiturates, morphine and apomorphine.* Schütz (1943) found that the activity of serum cholinesterase of human blood diminished after prolonged administration of barbiturates. Croft and Richter (1943) are reluctant to

attribute definite physiological significance to the changes observed under different conditions in blood serum cholinesterase, since its concentration is usually lower than that of blood corpuscles or of the tissue where most of the destruction of acetylcholine may be presumed to occur. Schütz found, however, that a prolonged administration of barbiturates in animals decreased not only the cholinesterase of serum but also that of the spinal cord and muscle. A single dose of the drugs sufficient to cause deep sleep did not have this effect. When barbiturates are given to epileptics the decrease in serum cholinesterase becomes maximal after three weeks, whereas the number of seizures becomes minimal within a few days; sudden withdrawal of the drug causes the number of seizures to rise sharply to a maximum whilst the cholinesterase is still at a low level, but seizures become less frequent again when the cholinesterase starts to rise (Schütz, 1944).

In *in vitro* experiments phenobarbitone does not inhibit the brain cholinesterase (Bernheim and Bernheim, 1936) which is however inhibited markedly by morphine (1 in 30,000) and apomorphine (1 in 48,000). Bernheim and Bernheim suggest that this action may contribute to some of their stimulating central effects. The common anesthetics inhibit cholinesterase only in much higher concentrations.

Confirmation is needed for the statement of Hoff and Pilcher (1935) that the acetylcholine content of rabbit's medulla, determined by acid alcohol extraction, rises from the normal value of 0.1 μg./g. to 10 and even 100 μg./g. on stimulation of the periarterial nerves in the wall of the aorta and carotid communis, and also for the statement of Haas (1939) that the acetylcholine content of the brain stem of rabbits, as determined by acid alcohol extraction, increases on intracisternal injection of large doses of *pilocarpine* and *adrenaline* but decreases on intracisternal injection of small doses of ergotamine; large doses were ineffective. Ergotamine decreased also the acetylcholine content of the cortex. No changes were observed after intravenous injections of these drugs. The observation of Diaz, Lorente, Moran and Simone (1941) that a substance like acetylcholine appears in the urine during attacks of *migraine* also needs confirmation.

Injections of large "convulsant" doses of acetylcholine have been used in the treatment of epilepsy, schizophrenia and other mental diseases but without success. For references see Cohen, Thale and Tissenbaum (1944).

9. THE PROPAGATION OF NERVE IMPULSES AND THE RELEASE OF ACETYLCHOLINE FROM CHOLINERGIC NERVE FIBRES. The suggestion has been made that acetylcholine is not only responsible for synaptic transmission but also for the propagated nerve impulse. Such a theory if proved would have a bearing on the chemical theory of synaptic transmission and the evidence in its favour as well as that against it must be included in this review.

Mention has been made of the findings that acetylcholine and cholinesterase are present in nerve fibres (see tables 1 and 2) and that the capacity to synthesize acetylcholine is not limited to the nerve endings but has to be attributed to the whole course of the fibre. Moreover there is evidence to show that the passage

of a nerve impulse releases acetylcholine not only at the endings of cholinergic nerves but also along their whole course; analogous conclusions apply to adrenaline and adrenergic nerves (Calabor, 1933; Bergamin, 1936, 1937, 1938; v. Muralt, 1937; Lissak, 1939). What is the physiological significance of these observations? There are three possibilities.

(1) No function is associated with the release of acetylcholine or of adrenaline along the fibre.

(2) The release of acetylcholine or of adrenaline modifies the nerve impulse.

(3) The release of acetylcholine is causally connected with and responsible for the nerve impulse. This possibility was envisaged by v. Muralt (1937). In a later publication (1943) he leaves open the question whether the release of acetylcholine is responsible for the spike potential or for the after-potentials, i.e., the recovery processes. Dale (1938) has stated the fundamental issue involved in this problem as follows: "if the liberation of a chemical mediator at a nerve ending should prove to be, not a process peculiar and limited to that ending, but merely a local intensification to ensure transmission to a contiguous cell, of a process which actually figures in the propagation of the impulse along the nerve fibre, we should have to make yet a further revision of our existing conceptions" and the particular difficulty in postulating a complete break in the nature of the processes concerned in conduction along fibres and transmission across synapses would then disappear," but only at the cost of a more fundamental change of conception concerning the nature of the propagated wave of excitation, than any which has yet been considered seriously." In fact, we should have to assume different mechanisms of nerve propagation for nerves which contain and for those which do not contain acetylcholine (Loewi and Hellauer, 1938; Hellauer, 1939).

The theory that acetylcholine is causally connected with the action potential of nerve fibres has recently been taken up again by Nachmansohn and his co-workers (Nachmansohn and Meyerhof, 1941; Nachmansohn, Coates and Cox, 1941; Nachmansohn, Cox, Coates and Machado, 1942, 1943; Nachmansohn, 1943; Fulton and Nachmansohn, 1943). They base their theory (1) on the presence of a high concentration of cholinesterase in nerves, which in the fibres of the squid is localised in the sheath, and (2) on the close parellelism they found between electromotor force and cholinesterase concentration in the electric organ of fishes. Their theory assumes that the discharge from the electric organ and the nerve action potential are fundamentally similar phenomena and that if the one is caused by the depolarising action of acetylcholine the other is initiated by a similar mechanism. The following considerations throw doubt on the justification of the interpretation given to their results.

(1) Nachmansohn and Meyerhof (1941) compared for the electric organs of the ray, torpedo and electric eel the voltage and number of plates per cm. with the concentration of cholinesterase and found a striking parallelism. This would mean that each plate has about the same concentration of cholinesterase. There are facts, however, which are not easily compatible with this view and suggest that the cholinesterase concentration is not the determining factor for the electromotor force (E.M.F.) but for the rate of subsidence

of the discharge, i.e., the disappearance of the released acetylcholine. For instance in the electric organ of the torpedo the action potential of the discharge has a quick descending phase. Eserine does not increase the potential, i.e. the E.M.F. is independent of the cholinesterase activity; but eserine lengthens the descending phase (Feldberg and Fessard, 1942). Such a lengthened discharge is the normal feature of the discharge from the ray untreated with eserine (Auger and Fessard, 1939) suggesting a lower concentration of cholinesterase than in the torpedo. The fact that the calculations of Nachmansohn and Meyerhof do not reveal such a difference makes it doubtful whether we are justified in laying much stress on the parallelism between voltage and cholinesterase concentration.

(2) The parallelism between E.M.F. and cholinesterase concentration is not so strict as it is claimed to be, as was shown by those experiments (Nachmansohn, Cox, Coates and Machado, 1942) in which actual determinations of E.M.F., number of plates and cholinesterase concentration were made from the same region of different organs even in the same species of fish. Discrepancies of the order of several hundred per cent were found when cholinesterase concentration was compared with either voltage of the discharge per centimeter or per plate.

(3) As the E.M.F. is dependent on the number, size and form of the plates and as the discharge is induced through nervous action only, it is not surprising that there should be some parallelism between E.M.F. and the concentration of a substance which like cholinesterase is either concentrated at the nerve endings or at the surface of the plates in close proximity to the endings, but not with substances which are not concentrated at this active region. Such a parallelism, however, does not justify correlating the size of the potential with the concentration of cholinesterase and then applying this parallelism to nerve fibres.

(4) There is no parallelism between the size of the action potential in nervous tissue and the concentration of cholinesterase (see table 2).

(5) The hypothesis that acetylcholine is intrinsically connected with the causation of the action potential in nerves does not take into account the fact that some nerve fibres are free from acetylcholine or contain traces only (see table 1). These fibres are either unable to synthesize acetylcholine or they have this property to a very small degree. If we are thus forced to assume that different substances are responsible for the propagation of nerve impulses in different fibres, then the presence of cholinesterase in those fibres which are not cholinergic would prove that its presence is of no special significance for the causation of the action potential. It is worth while remembering the wide distribution of cholinesterase in non-nervous tissue emphasized by Torda (1942).

(6) Even in cholinergic nerves impulse conduction and acetylcholine content or the ability to synthesize it do not run parallel. In degenerating nerves the nerve fibres are still able to conduct impulses at a time at which they have lost their acetylcholine content as well as the ability to synthesize it (Feldberg, 1943).

(7) Action potentials are not confined to nervous tissue. The action potentials of skeletal muscles would have to be explained on entirely different lines, since the muscle fibres contain little acetylcholine or cholinesterase.

(8) There is no evidence that acetylcholine, which excites motor endplates, electric plates and nerve cells, probably by a depolarising action, has a similar effect on nerve fibre. Fraser (1867) found no effect of the active principle of the calabar bean on nerve fibres which would warrant such a conclusion, and Cowan (1938) stated that treatment of a frog's nerve with prostigmine or eserine produced practically no change in the action current and only slightly modified the responses of nerve muscle preparations to stimuli of different frequencies. More recently Cantoni and Loewi (1944) have shown that in frogs a subcutaneous injection of eserine did not affect the propagation of impulses in the peripheral nerves despite the fact that their cholonesterase activity was inhibited. Riesser and Neuschlosz (1921) obtained no excitation of motor nerves by immersing them in acetylcholine solutions. Bronk (1939) showed that the injection of acetylcholine into a perfused sympathetic ganglion caused a vigorous discharge from the ganglion cells but initiated no impulses in the preganglionic fibres, although the presynaptic neurons were accessible to exciting

agents in the perfusion fluid, as shown by their response to sodium citrate. Recently Lorente de Nó (1944) has shown that acetylcholine even at a concentration of 0.1 M does not depolarise or excite the nerve fibre or prevent the conduction of nerve impulses. In fact whereas acetylcholine may be the sole effective stimulus for the discharge of electric plate being inexcitable to electric or mechanical stimuli, the nerve fibre can be excited by a number of stimuli but apparently not by acetylcholine.

In conclusion it may be said, therefore, that apart from the fact that the nerve impulse releases acetylcholine from cholinergic nerves and may even be associated with its resynthesis, there is at present no evidence to warrant the conlusion of a further intrinsic relationship between nerve impulse and acetylcholine metabolism. It is conceivable that the released acetylcholine may influence the excitability or conductivity of cholinergic fibres and may have some modifying influence on the action potentials. Only further research carried out, not on electric organs but on nerve fibres, can give an answer to this problem. In this connection it is interesting to note that eserine in relatively low concentration affects the membrane potential of nerve fibres during anoxia and during the following period of oxidative recovery (Lorente de No, 1944).

## CONCLUSIONS

The present position of the theory of acetylcholine as central transmitter is all but settled. The acceptance or rejection of the theory will depend upon the relative value which is to be attached to the various findings reviewed in the foregoing pages and upon what kinds of evidence are regarded as decisive. No reference has been made to the short central synaptic delay or to the short refractory period in considering transmission at central synapses and for the following reason: the shortness of these time intervals does not in itself provide evidence either for or against the theory that acetylcholine is the transmitter substance at central synapses. If there is sufficient other evidence available in support of the acetylcholine theory these time intervals demonstrate only (a) the rapidity with which the acetylcholine is released and acts, which must exclude any process of diffusion from the site of liberation to the site of action, and (b) the rapidity with which the acetylcholine is inactivated within the refractory period, which probably excludes any but enzymatic processes.

All the evidence in favour of acetylcholine as the central transmitter substance has been obtained by methods previously applied to the peripheral nervous system and the arguments in favour of the theory must necessarily be based on the same kind of reasoning as was applied to the peripheral nerve effects. The presence of acetylcholine and of cholinesterase in central nervous tissue, the ability of such tissue to synthesize acetylcholine and to release it under certain conditions, the central effects of acetylcholine and of eserine, all provide strong evidence in favour of the theory that the transmission across a number of synapses in the central pathway of autonomic and motor neurons occurs through the mediation of acetylcholine. In fact the evidence in some instances is as good as or better than that available for the transmission by acetylcholine across ganglionic synapses or from motor nerves to motor endplates. On the other hand there is little evidence in favour of such a mechanism of transmission across

many other central synapses, and there are some facts which are at present difficult to reconcile with the theory of acetylcholine as the universal central transmitter.

(1) The acetylcholine content of the brain decreases with the higher development of the species and this decrease can scarcely be attributed to a greater proportion of supporting tissue in more highly developed brains. Nor can it be accounted for by the assumption that a lowering of the store of acetylcholine is associated with increased ability to synthesize it, an explanation which has been put forward to explain the difference in the peripheral and the central nervous system. The ability to synthesize acetylcholine and the concentration of cholinesterase appear to diminish together with the decrease of acetylcholine in the more highly developed brains. It is natural to suggest that the transmission by acetylcholine may be a more primitive type of central transmission, the importance of which receded in higher animals, and which is retained in them at certain central synapses only. Many facts, however, cannot be explained in this way and our knowledge at present is too scanty to justify a definite statement of this kind; for instance, the sensitivity of the nerve cells may increase in higher animals.

(2) The sensory fibres contain no acetylcholine or only traces; similarly they can only synthesize it in small amounts if at all. How then are we to explain the transmission by acetylcholine of impulses from these fibres to the cells in the spinal cord? We should have to assume as pointed out by Loewi and Hellauer (1938), that the sensory fibres at their endings acquire new properties, an assumption for which there is at present no experimental evidence.

(3) Whereas the effects of acetylcholine and of eserine on the motor cortex and in the spinal cord, as well as the release of acetylcholine from the cord during increased reflex activity, suggest synaptic transmission by acetylcholine in the central motor pathway, the fibres of the pyramidal tract contain extremely small amounts of acetylcholine. This makes it difficult to consider them as cholinergic fibres.

(4) The cerebellum is rich in synapses but again contains only traces of acetylcholine and its ability to synthesize acetylcholine is small. Against this fact must be placed the one experiment of the effectiveness of eserine in a large concentration on local application to the cerebellar cortex.

If we must assume that acetylcholine is not the universal central transmitter then our conception concerning its rôle in the central nervous system will be influenced greatly by the view we take concerning the transmission across those synapses not effected by acetylcholine. Must we assume different transmitter substances in the central nervous system or must we assume that, in some, excitation is effected by the circulating currents from the presynaptic terminations? In that case both transmission processes might be involved in the transmission across a single synapse, the relative importance of the one or the other varying at different synapses. Bronk (1939) argues for such a pluralistic theory even with regard to transmission across sympathetic ganglia. It is fruitless at present to try to force the issue, as we do not know if nerve cells can be activated solely by the circulating currents in their synaptic terminations.

Lorente de Nó (1935) found that a subliminal afferent volley and a subliminal induction shock applied apparently directly to a central motor neuron can sum to produce threshold excitation. From this observation he concludes that cell body and dendrites with their synaptic surfaces are electrically excitable and that therefore the action current is the central synaptic transmitter. There are, however, experiments which suggest that the cells of sympathetic ganglia, at least, cannot be excited in this way. We have mentioned the inability of the nerve impulse to cross the sympathetic synapse on reaching the ganglion in the early stage of nerve degeneration when acetylcholine has nearly disappeared from the nerve and its ability to synthesize acetylcholine has been lost. Further proof is found in the regeneration experiments of Langley and Anderson (1904). As Dale has pointed out, their results show that only cholinergic fibres, and not sensory or adrenergic fibres, are able to make functional connection with a sympathetic ganglion. If it had been shown that the regenerating adrenergic or sensory fibres had reached the ganglion and had conducted impulses right up to the cells without however exciting them, these experiments would have furnished strong proof that the electrical variation of the nerve impulse itself is insufficient for excitation.

Similar experiments carried out with skeletal muscles have given conflicting results. In mammals the results were the same as in the ganglion, that only cholinergic fibres were able to enter into functional junction with the motor endplates (Langley and Anderson, 1904). On toads, however, Weiss (1934, 1935) obtained different results. When the central end of a sensory root was allowed to grow into a denervated striped muscle electrical stimulation of the sensory nerve produced muscular contractions in a few experiments, but the impulses set up in these nerves by natural stimulation from the skin never had this effect. It is thus doubtful that real sensory fibres had made functional contact with the muscle. However when the peripheral end of the sensory nerve was allowed to grow into the denervated muscle it always contracted on electrical stimulation of the regenerated sensory nerve. As sensory nerves may contain a few cholinergic fibres we do not know if the circulating current of the impulse itself had the excitatory effect. The acetylcholine content of the regenerating fibres has not been determined. A further difficulty in assessing the bearing of the experiments of Weiss on the problem of the excitability of motor endplates lies in the fact that the regenerating nerve fibres ended on the muscle fibre and not on specified plates. Experiments on endplates have been performed by Buchtal and Lindhard (1939) in muscles of lizards. According to their results we must assume two boundary faces with different properties of excitability, a boundary between the nerve ending proper and the sole, and a boundary between the sole and the muscle fibre. The latter is certainly excitable electrically but the former may possibly be excitable to acetylcholine only. At least when it is blocked by excess of acetylcholine to the action of acetylcholine, it is also unaffected by the nerve impulse. The electrical inexcitability of denervated plates of electric organs of fishes also suggests electric inexcitability of motor endplates at the boundary facing the nerve terminations.

The main danger at present for the theory of central transmission by acetyl-

choline is not the difficulties which oppose it, but the attempt to base the theory on single items of evidence. It is possible that a completely new approach must be found in order to settle the problem. One new approach might be provided by injecting large amounts of purified cholinesterase into animals or organs and to study the effect on the transmission process. Mendel and Hawkins (1943) have applied this method to the light reflex but the effect of the cholinesterase in their experiments was, as they themselves consider, mainly on the acetylcholine released from the parasympathetic endings in the pupil.

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# GENETICS AND METABOLISM IN NEUROSPORA

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Within recent years there has been a noticeable shift in emphasis in the field of genetics away from studies on the mechanism of gene transmission to investigations in such subfields as population genetics, the physical and chemical nature of genes, the mechanism of gene mutation, and the manner of gene action in development and function. The last of these topics is often referred to as physiological or biochemical genetics. It is this branch of genetics as it is approached through studies on the fungus *Neurospora* with which the present review is primarily concerned.

There are a number of instances known in which the influence of specific genes can be referred to more or less well known chemical processes. Among these are gene-antigen relations, flower pigmentation, specific abnormalities in phenylalanine-tyrosine metabolism in man, mating reactions in protista, pigmentation in animals, sugar fermentation by yeasts, atropine esterase activity in rabbits, and a number of others [see reviews by Wright (66, 67), Lawrence and Price (30), Haldane (22, 23), Sonneborn (50), Ephrussi (16), and Beadle (3) for details and references]. Consideration of examples such as these has led to the suggestion that there exists for most genes a one-to-one relation between gene and specific reaction (5, 27). This implies that the primary action of a gene is in the control of a specific reaction.

With this concept in mind the general problem has been approached in *Neurospora* in a manner somewhat different from that usually followed (5). If it is true that for every biochemical reaction, or perhaps more precisely every enzymatically catalyzed reaction, there is in primary control one specific gene, it should be possible to select a reaction which it is desired to investigate and by means of induced mutation discover the gene in primary control of it. Some categories of reactions are obviously simpler to work with than others and a deliberate selection has been made of those in which the products can be supplied from outside the organism. Reactions leading to the synthesis of amino acids and vitamins, for example, fall into this classification. An organism that cannot synthesize thiamin can survive if this vitamin is supplied to it from an external source. Therefore, if a sufficiently large number of gene mutations were induced in an organism which could synthesize this substance, some of them might be expected to result in a loss of the ability to make this specific compound. In this way it should be possible to convert an organism originally independent of an external source of thiamin into one dependent for its existence on such a supply. Experimental control can be had by regulating the amount supplied of this vitamin which we are already fairly certain is necessary for all organisms.

The study of such reactions necessitates the choice of an organism in which the

raw materials supplied are completely under the control of the investigator. This limitation immediately excludes the use of all higher plants and animals since these cannot readily be grown on chemically defined media and under aseptic conditions. It suggests the use of micro-organisms. But all bacteria and blue-green algae are out of the question because, without sexual reproduction, genetic control in the usual sense cannot be had. Certain green algae suggest themselves, but breeding technics in these are usually not simple. This is likewise true of protozoa, and they suffer from the additional disadvantage of having complex and not completely understood dietary requirements. Fungi, on the other hand, seemed more promising. The ascomycete *Neurospora* has a life cycle almost ideally suited to genetic studies, can be grown under aseptic conditions, is not unreasonable in its space requirements, and has been studied rather extensively from a genetic standpoint (10–14, 32, 49). Its one disadvantage at the time it was first considered was that its growth factor requirements were not known. Investigation soon showed, however, that all commonly cultured species can be grown on a medium containing inorganic salts, an inorganic nitrogen source, any of a number of carbon sources such as glucose, sucrose, starch or fat, and the growth factor, biotin (5, 7). At the time of these studies the chemical nature of biotin was not known but it was nevertheless available for experimental purposes either as a concentrate or as pure crystalline material. Fortunately, its structure is now known and synthetic material has become available.

I. The organism. A. *Historical.* *Neurospora*, known in its imperfect or non-sexual stages as *Monilia*, was called to the attention of science over one hundred years ago as a pest in bakeries. It grows prolifically on bread, producing masses of salmon colored conidia which are dispersed in dense clouds when disturbed. Before the days of mold inhibitors in commercial bread, a bakery infested with *Neurospora* was in for serious difficulties. On this account it was the subject of several scientific inquiries in the last century. (See Shear and Dodge, 49, for accounts of these.) With the discovery of the sexual stage by Shear and Dodge and with the finding that the ascospores can tolerate remarkably high temperatures (see below) it became clear that a possible reason for the difficulty of controlling the mold lies in the fact that the sexual spores are able to withstand the temperature attained in the interior of loaves of baking bread.

The genus *Neurospora* is widely distributed in the tropics where it frequently is a first colonizer of burned-over areas. Vorderman (62a) states that in the East Indies, following burning in connection with volcanic eruptions, *Neurospora* completely overgrows the charred remains of vegetation, producing great masses of brilliant conidia, and creating a most bizarre appearance. The resistance of its sexual spores to heat of course gives it a great selective advantage under these conditions.

Near the close of the last century the well-known Dutch botanist, F. A. F. C. Went, was stationed in Java. He was interested in fungi used for technical purposes and his attention was called to *Monilia sitophila*, now known as *Neurospora sitophila*, which was used by the Javanese in making a kind of delicacy

known as "ontjom." According to Heyne (23a), Vorderman (62a) and Went (64) ontjom are prepared from peanut meal from which the oil has been expressed. Layers of this residue are spread on banana leaves and seeded with *Neurospora* spores. After several days' growth of the fungus, during which the peanut meal is fermented and brilliant orange conidia are produced in profusion, the ontjom are ready for consumption.

Prof. F. A. F. C. Went undertook to study the "ontjom fungus" from a physiological standpoint. He encountered serious difficulty, however. At the high humidities of the tropics the fungus has the discouraging habit of growing rapidly through the cotton stoppers of the containers in which it is cultured. The investigations were deferred until his return to Holland. There "*Monilia sitophila*" could be cultured readily. He described it in detail (64) and carried out various physiological studies in which he investigated its growth in chemically defined media containing various carbon and nitrogen sources and in which he made a remarkably thorough investigation of the ability of the organism to produce enzymes extracellularly and in response to specific substrates (63, 64, 65).

In all of Went's investigations biotin was supplied inadvertently as an impurity and from the data on dry weights of mycelia produced was evidently a limiting factor in most instances. In a discussion of the results of experiments in which raffinose was added to media containing other sugars, Went came close to discovering that some accessory factor (biotin) was necessary for the growth of *Neurospora*. He presents an elaborate discussion of the disproportionate effect of small amounts of raffinose, and as one reads this, it is difficult to see why the idea of an impurity in the raffinose did not occur to him. It is, of course, easy to see the explanation now, but one must remember that Went wrote in 1901, about the time Wildiers suggested accessory growth factors for yeast and many years before the concept of vitamins, as we now know it, took definite form.

The year 1927 marks the birth of the genus *Neurospora* (literally "nerved spores") and the beginning of its use in genetic studies. In this year Shear and Dodge (49) published their account of the life histories of members of the genus, including the sexual stages. They proposed the generic name and described the heterothallic species, *sitophila* and *crassa*, and the homothallic species, *tetrasperma*. Dodge immediately recognized the advantages of *Neurospora* for genetic studies and undertook a series of investigations which have been continued without interruption since (10–14). He determined the manner of sex inheritance, demonstrating that segregation for the gene pair concerned might occur at either the first or second meiotic division in *N. sitophila*. Albinism (more precisely, failure to form conidia) in *N. stiophila* was shown to be inherited in a simple fashion with genetic segregation occurring in either the first or second meiotic divisions.

With characteristic enthusiasm Dr. Dodge pointed out to Prof. T. H. Morgan, then at Columbia University, the advantages of the new material for genetic studies. Morgan carried a collection of cultures to the California Institute of Technology in 1928 where he maintained them for a period of about a year.

They were eventually turned over to Dr. C. C. Lindegren who has since made substantial contributions to our knowledge of the genetics of the organism.

B. *Life cycle.* An understanding of how *Neurospora* is used experimentally in biochemical genetic studies necessitates an appreciation of certain features of its life cycle. These are shown diagrammatically in figure 1 for heterothallic species which are most convenient for genetic studies. The vegetative hyphae are cylindrical tubes with septae at intervals. Each segment is multinucleate with perhaps as many as twenty nuclei. Each septum has a central perforation large enough to allow passage of hyphal nuclei. Thus the mycelium is essentially coenocytic. The individual nuclei are haploid, carrying a single set of seven chromosomes (McClintock, personal communication, 38).

On a suitable medium, myclia grow rapidly by a process of elongation of individual hyphae at their tips and through profuse hyphal branching. Some few days after growing over a suitable medium, chains of asexual spores are cut off at the tips of aerial hyphae (conidiophores). Most of these conidia contain several nuclei although an occasional one may be uninucleate. A second type of asexual spore known as a microconidium is also produced (13). According to Lindegren and Lindegren (33, 34) microconidia are uninucleate. Vegetative reproduction therefore occurs in three ways: 1, by means of conidia; 2, through microconidia, or 3, by way of hyphal or mycellial fragments.

*Neurospora crassa* and *N. stiophila* are heterothallic, i.e., strains of two sexes exist. These are morphologically identical so far as we know but are physiologically distinct as shown by their mating reactions. These two sexes are usually kept in separate cultures in the laboratory. They are designated *A* and *a* types (or A and B according to earlier nomenclature of Dodge, or + and − in Lindegren's terminology).

If spores or hyphae of the two sexes are placed together on a suitable medium, fusion of hyphae occurs and sexual fruiting bodies known as perithecia are initiated. Within these perithecia, fusion between nuclei of opposite sexes occurs in structures known as ascus hooks. The resulting diploid zygote nucleus undergoes two meiotic divisions the details of which follow the conventional pattern (10, 38). These occur in such a way as to give four haploid nuclei lying along the axis of the spore sac or ascus. There follows a mitotic division of each of the four primary meiotic products, giving a row of eight nuclei each with seven chromosomes. Each is included in a sexual spore (ascospore) which is an ellipsoidal, black body about 28 $\mu$ long with longitudinal ridges on its surface (49). A further mitotic division follows by which the ascospores become binucleate, their condition at maturity.

On cornmeal agar or other suitable media, both *A* and *a* strains of *N. crassa* or *N. sitophila* produce "protoperithecia" (13, fig. 1). These are spherical masses of hyphae from which specialized hyphae known as trichogynes protrude. In the absence of hyphae or spores of the opposite sex the proteperithecia remain as infertile bodies. If conidia or microconidia of the oppoiste sex come in contact with the trichogynes, connecting pores develop by which the contents of the conidia enter the trichogyne (1). The nuclei of the conidia migrate

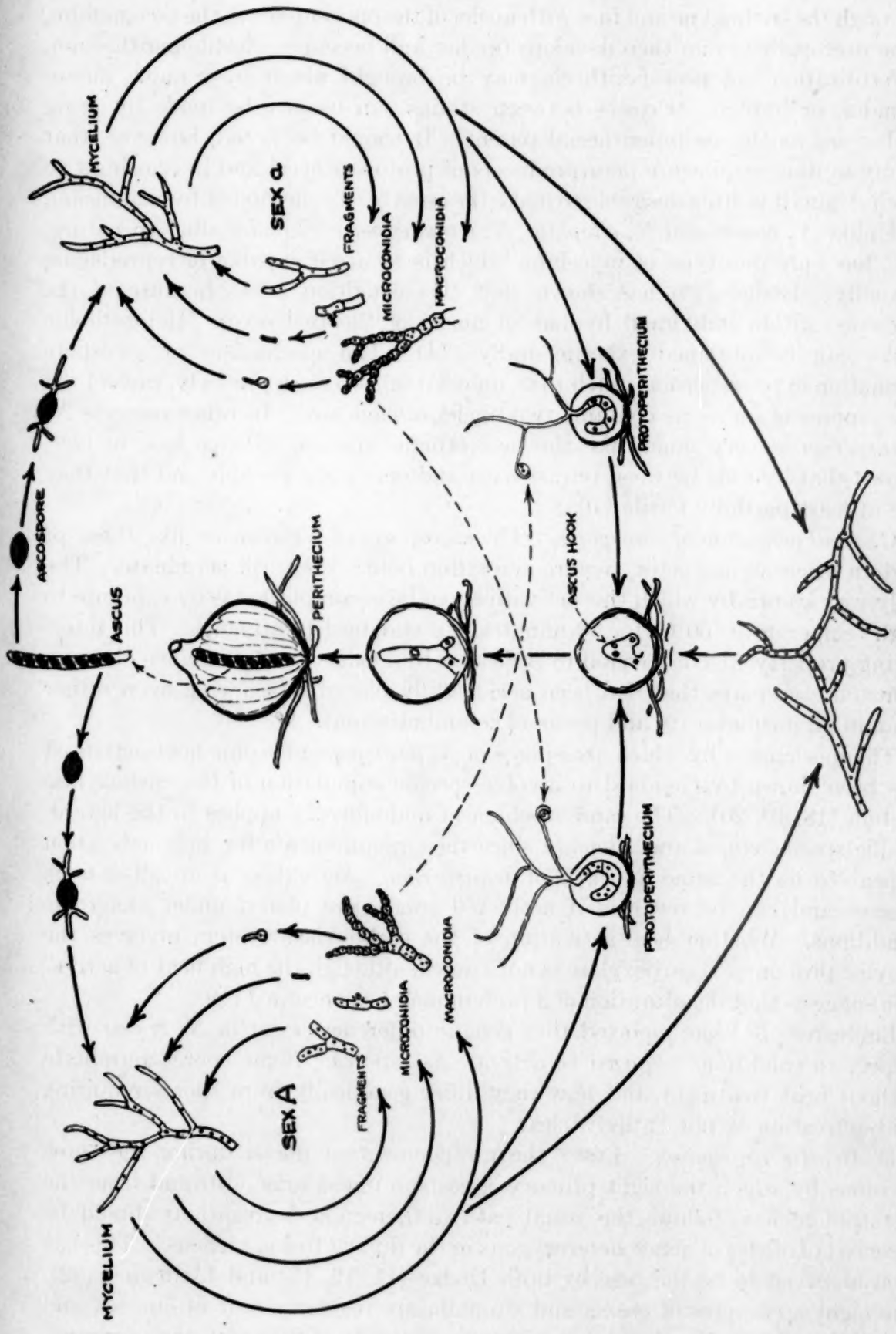


Fig. 1. Diagrammatic representation of life cycle of *Neurospora crassa* (or *sitophila*).

through the trichogyne and fuse with nuclei of the opposite sex in the ascogonium. The protoperithecium then develops further and becomes a fertile perithecium. "Fertilization" of protoperithecia may be brought about by conidia, microconidia, or hyphae. Crosses between strains can usually be made by using either sex as the protoperithecial parent. It should be noted, however, that many mutant strains are poor producers of protoperithecia and in crossing two such strains it is often desirable to make the cross by the method of hyphal fusion.

Unlike *N. crassa* and *N. sitophila*, *N. tetrasperma* is "homothallic" in nature, i.e., has only one type of mycelium which is by itself capable of reproducing sexually. Dodge (10) has shown that this condition arises because of the presence within individual hyphae of nuclei of the two sexes. Heterothallic races can be obtained experimentally (14). The mechanism of ascospore formation in tetrasperma is such that, unless it fails to work precisely, each of the four spores of an ascus contains two nuclei of each sex. In other respects *N. tetrasperma* is very much like the heterothallic species. Dodge has, in fact, shown that hybrids between tetrasperma and crassa are possible and that they are at least partially fertile (10a).

C. *Heat activation of ascospores.* The ascospores of *Neurospora*, like those of certain other ascomycetes, require activation before they will germinate. The only way known by which this activation can be accomplished is by exposure to high temperature (60°C. for 30 minutes is a standard treatment). This interesting property was discovered in *Ascobolus* by Dodge through chance observations on ascospores that had been accidentally placed in a drying oven rather than in an incubator (9, and personal communication).

The mechanism by which ascospores of *N. tetrasperma* become heat-activated has been shown by Goddard to involve specific stimulation of the carboxylase system (18, 19, 20). The same mechanism undoubtedly applies to the heterothallic species *crassa* and *sitophila* since their requirements for heat-activation appear to be the same as those of *tetrasperma*. Activation is an all-or-none process and can be reversed if activated spores are placed under anaerobic conditions. Whether heat-activation of the carboxylase system involves the enzyme protein or cocarboxylase is not known, although the high heat of activation suggests that denaturation of a protein may be concerned (19).

Lindegren (31) has reported that genetic differences exist in *N. crassa* with respect to conditions required to activate ascospores. Some spores germinate without heat treatment, but how they differ genetically from spores requiring heat-activation is not entirely clear.

D. *Genetic segregation.* From the arrangement of nuclei during the three divisions by which the eight primary ascospore nuclei arise (10) and from the fact that meiosis follows the usual path (38), regular segregations should be observed of alleles of genes heterozygous in the diploid fusion nucleus. This has been observed to be the case by both Dodge (11, 12, 13) and Lindegren (32). The eight ascospores of crassa and sitophila are regularly four of one sex and four of the other. Numbering ascospores from the tip of the ascus, their arrangement with regard to sex has been shown to be any one of the following:

A A A A a a a a
a a a a A A A A
A A a a A A a a
A A a a a a A A
a a A A A A a a
a a A A a a A A

The first two arrangements represent first division segregations of the two alleles of the sex gene (or possibly a differential segment of a chromosome), while the remaining four types show second division segregation of the two

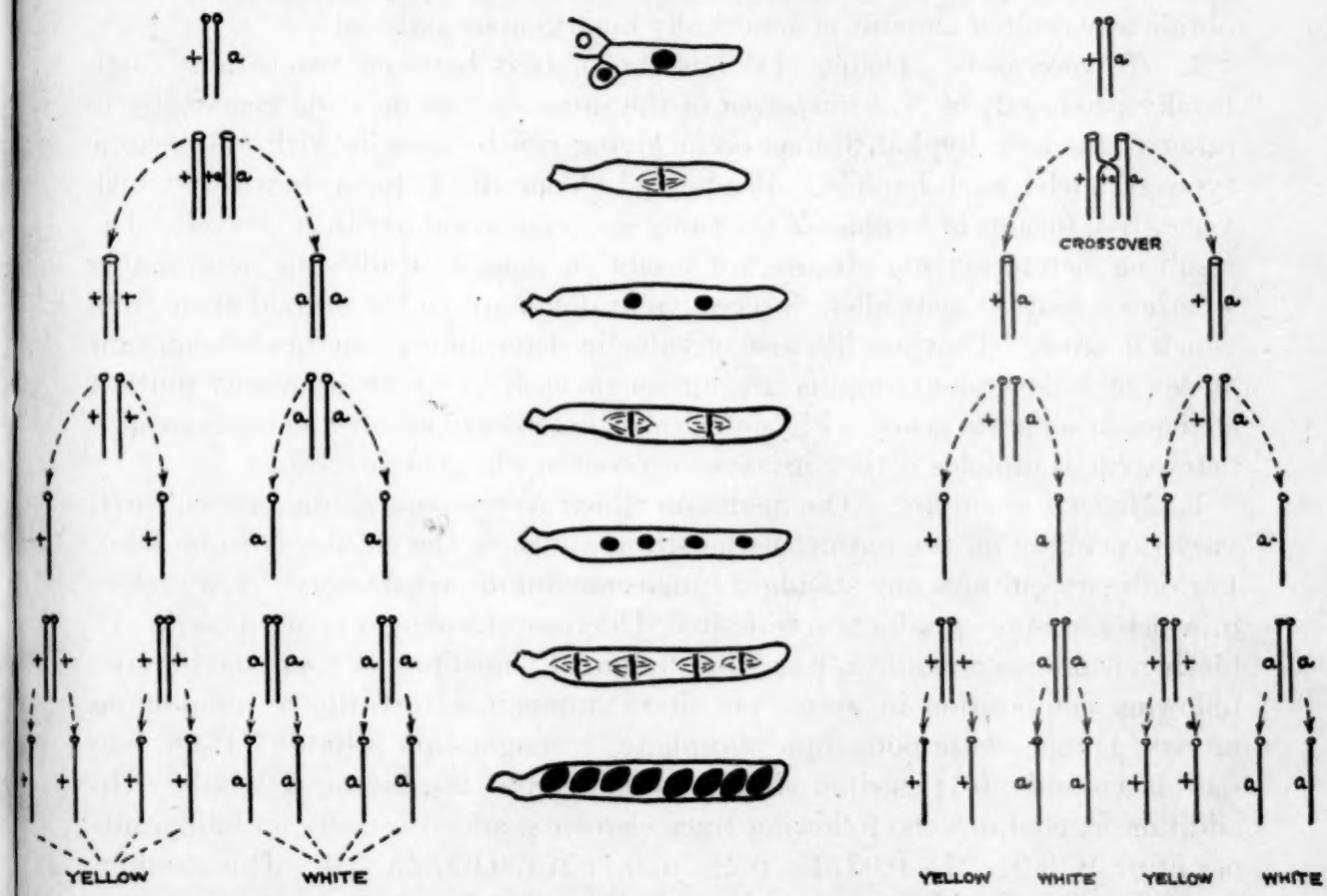


Fig. 2. Nuclear and chromosome history in developing ascus from cross of wild type and albino strains. Nuclear divisions shown in center column. Left, chromosome basis of first division segregation. Right, chromosomal basis of second division segregation. Two alleles of segregating gene are designated + (normal allele) and *a* (mutant allele).

alleles. The chromosomal basis of these two types of segregation is shown in figure 2 in which segregation of yellow and albino strains in *N. crassa* is represented.

The occurrence of a chiasma between the centromere and a segregating gene pair results from physical exchange of corresponding segments of homologous chromosomes and is expressed in the final arrangement of spores as second division segregation. The frequency of formation of such chiasmata between the centromere and a given gene locus is dependent on the distance of the gene from the centromere. Hence frequency of second division segregation is a

measure of distance of the gene pair concerned from the centromere. This distance can be determined in terms of the same cross-over or map units by which gene arrangements are expressed in higher plants and animals.

From a genetic standpoint, an organism like *Neurospora* has several advantages. In the first place, the haploid phase is the one usually studied. There are therefore no complications due to dominance, and the ratios of types measure directly the proportions in which meiotic products occur. Secondly, since all products of meiosis are recovered, basic ratios are mechanically determined and are therefore not subject to sampling errors. Finally, strains can be multiplied rapidly by vegetative means and it is thereby possible to obtain any desired amount of genetically homogenous material.

E. *Heterocaryosis.* Dodge (14) has shown that between two strains (artificially produced) of *N. tetrasperma* of the same sex but differing genetically in other characters, hyphal fusions occur giving rise to mycelia with two genetic types of nuclei, each haploid. Beadle and Coonradt (4) have shown that such vegetative fusions of hyphae of the same sex occur regularly in *N. crassa*. The resulting heterocaryotic strains are useful in genetic studies in determining whether a mutant gene allele is recessive or dominant to the normal allele from which it arose. They are likewise of value in determining whether two mutant alleles of independent origins are alleles of each other or represent mutant changes in separate genes. Physiologically, heterocaryons are the equivalent of heterozygous diploids if they involve two component genetic strains.

F. *Methods of culture.* The media on which *Neurospora* strains are cultured vary depending on the nature of the strain and how the culture is to be used. For ordinary cultures any standard fungus medium is satisfactory. For crosses in which ascospore production is desired, Difco cornmeal agar is often used. In biochemical experiments a basal or "minimal" medium is used having the following composition in grams per liter: ammonium tartrate, 5; ammonium nitrate, 1; monobasic potassium phosphate, 1; magnesium sulfate (7 $H_2O$), 0.5; sodium chloride, 0.1; calcium chloride, 0.1; sucrose, 20; biotin, $5 \times 10^{-6}$. In addition, it contains the following trace elements, added as salts, in milligrams per liter: B, 0.01; Mo, 0.02; Fe, 0.2; Cu, 0.1; Mn, 0.02; Zn, 2.0. This medium may be used in liquid form or made semisolid with 1.5 per cent agar. If agar is added, care should be taken that materials undesirable in a given experiment are not inadvertently added with it. Especially washed agar is often necessary (44, 45). For particular experiments the above minimal medium may be supplemented with desired compounds, or otherwise modified.

Ryan, Beadle and Tatum (45) have made a detailed study of growth on solid medium in "growth tubes." In these, progression of a mycelial frontier along a horizontal agar surface in a glass tube can be measured conveniently. A similar quantitative measure of growth on agar surfaces in petri plates has been investigated (62). Quantitative measures of growth in liquid medium can be made by weighing dried mycelial pads from Erlenmeyer culture flasks of any desired size, e.g., 125 ml. flasks containing 20 ml. of medium (26).

Single ascospore cultures, necessary for genetic control, are readily obtained by transferring ascospores on small blocks of 3 per cent agar to small test tube slants.

Isolations may be made either at random or in order from individual asci. Any conidia, microconidia, or bits of mycelium inadvertently transferred with an ascospore are almost invariably killed at the high temperature at which ascospores are activated (30 min. at 60°C.). In order to allow ascospores to age before heat treatment and to make doubly sure that no vegetative spores or fragments contaminate single ascospore cultures, it is often desirable to flood individual ascospores with a 1.5 per cent solution of sodium hypochlorite. This is effective in killing both vegetative spores and mycelial fragments (Beadle, unpublished observations).

II. Production of mutant strains deficient in specific synthesis. The theory and practice of producing and selecting mutant strains of Neurospora in which specific synthetic reactions are blocked have been described by Beadle and Tatum (5, see also reviews 27, 57, 58). On the assumption that all enzymatically catalyzed reactions bear a rather direct relation to specific genes, it should be possible, by producing gene mutations at random in a sufficient number of strains, to select those in which almost any synthesis is interrupted.

It is known that ionizing radiation such as x-rays, gamma-rays, and neutrons, produce relatively permanent changes in genes. These may represent physical destruction of genes, reversible inactivations or other changes (40). Mutations are likewise produced following absorption of ultraviolet radiation. These appear to differ at least statistically from those produced by ionizing radiation in that they involve a smaller proportion of physical losses and physical rearrangements of chromosome parts and a greater proportion of potentially reversible gene changes (51, 52). It should be recognized that valid deductions can be made as to the rôle played by a given gene whether we determine this rôle by experimentally eliminating the particular gene or by partially or completely inactivating it while leaving its power of self-duplication unimpaired.

The procedure employed in producing "biochemical mutants" in Neurospora (5, 27) involves raying conidia either dry (with x-rays or neutrons) or suspended in liquid minimal medium (ultraviolet). X-ray dosages up to 100,000 r units may be applied to dry conidia. Ultravioletet radiation from a Westinghouse sterilamp which emits some 90 per cent of its energy at 2537 Å, has been used. This wavelength is near the maximum absorption of nucleic acid which Stadler and Uber (52) and others have shown to coincide with the maximum sensitivity of genes to ultraviolet-induced mutation. At this wavelength some conidia will tolerate 15,000 ergs per $mm^2$. Following treatment, conidia are applied to protoperithecia of the opposite sex. From the mature perithecia so produced ascospores are taken—one ascospore per perithecium to avoid obtaining the same mutant twice. These are grown in small individual test tube cultures. Since mutations are induced prior to meiosis, the vegetative descendents of a single ascospore are genetically homogenous, barring further spontaneous mutation. This would not be so if mature ascospores or conidia were treated and their direct vegetative descendents investigated. It may not be true if the direct descendents of uninucleate microconidia are used (33, 34), since it is possible that chromosomes may be effectively double even in a haploid nucleus.

Single ascospore strains are grown on a medium made as complete as possible

in vitamins, amino acids and other growth factors so that any induced deficiency in synthetic ability will be compensated for by a component of the medium. After establishment on "complete" medium, vegetative transfers (conidia) are made of strains to a minimal medium. If normal growth occurs on various minimal media (with carbon and nitrogen sources varied and with other factors such as pH and temperature varied), the culture is considered to be like the original wild type strain and is discarded. If, however, growth fails to occur on one or more minimal media, a mutation blocking some essential reaction is assumed to have taken place. By making a systematic series of tests on minimal media supplemented in various known ways, it is possible to determine something about the nature of the altered growth requirements of a particular strain. For example, if growth occurs on a minimal medium supplemented with pyridoxin but not on media in which this vitamin is absent, it is assumed that some essential reaction in the synthesis of this compound has been interrupted.

Once mutant strains have been identified it is a simple matter to determine how they differ genetically from the original strain. Crosses with wild type of the opposite sex are made. From a number of asci the eight ascospores are taken in order and grown in individual cultures on a medium capable of supporting growth of both mutant and wild-type strains. After cultures have become established on this medium, vegetative transfers to a suitable minimal medium are made. If from each set of eight spores four strains are obtained which fail to grow on unsupplemented minimal medium and four capable of doing so, and if the normal and mutant spores are in the expected arrangements, we are justified in concluding that the mutation involved an alteration of a single point in a chromosome, i.e., was a single gene change or single gene loss. There is some possibility that simultaneous loss of several adjacent genes would give the same genetic result as loss or inactivation of a single gene but it does not seem probable that such a multiple defect could be compensated for by supplementing the minimal medium with a single chemical compound.

Some 60,000 single spore strains, the parents of which were treated with x-rays, neutrons, or ultraviolet, have been tested on various minimal media (27). It is not possible at present to give an exact figure for the percentage of biochemical mutants among these, since many have been classified tentatively only and require further study. Nor is it possible to say how many genes have been involved in mutants of this class since recurrences of the same mutation are often found. Without detailed genetic and biochemical tests these cannot be distinguished from mutations in separate genes concerned with different steps in a sequence of reactions leading to a particular end product. It is probably a fair estimate, however, that at least 100 genes affecting vital reactions have been mutated (27). The majority of these involve the synthesis of vitamins, amino acids or nucleic acid components. However, it is evident both on theoretical grounds and in experimental practice that the types of mutants detected will depend on the conditions under which the selection is made. For example, yeast extract is a constituent of the usual "complete" medium. This is known to contain substances specifically inhibitory to certain mutants and it is clear

that a medium containing it does not favor the selection of these mutants. Furthermore, it has been shown that specific mutant types grow normally on a minimal medium at one pH but not at another (53, 55). Their detection obviously depends on the use of a minimal medium of a suitable hydrogen ion concentration. After the detection of mutant strains showing defects in synthetic ability, the classification of these in terms of the reactions concerned depends on key compounds being both known and available for use. There is a strong tendency to concentrate on those mutants that are most easily classified and this of course means selection of those concerned with synthesis of compounds of recognized nutritional significance. Too much weight should not therefore be assigned to the fact that most of the mutants reported involve these compounds. Even making allowance for these biasing factors, however, most of the clear-cut mutants have been shown to involve synthesis of known compounds. From a statistical standpoint this means that there are probably not many more members of the B-complex of vitamins to be discovered. Similar reasoning indicates that the majority if not all the natural amino acids of nutritional importance are known.

III. Arginine synthesis. Srb and Horowitz (55) have analyzed fifteen mutant strains, the growth requirements of which are met by arginine. Seven of these proved to be genetically distinct. The remaining eight represent recurrent mutations of genes involved in the first seven.

These arginine mutants may be used to illustrate the methods of demonstrating genetic identity or non-identity of mutant alleles. If two mutant strains result from mutation of the same gene, a cross between them will yield only mutant offspring. Furthermore, a heterocaryon between them will show the same growth requirement as the mutant strains—it is the physiological equivalent of a homozygous diploid in higher plants and animals.

If, on the other hand, two strains requiring arginine or a related compound for normal growth result from mutations of different genes, the cross between them will yield both mutant strains and strains which like the original wild type grow in the absence of arginine. If the two mutant genes are closely linked, there will be few wild type offspring produced; if they are carried in separate chromosome pairs, wild type descendants will be expected to constitute 25 per cent of the total. In certain types of asci it is possible to identify double mutant spores even though physiologically these may not be distinguishable from one of the component single strains. For example, if $a_1$ and $a_2$ represent mutant alleles of two genetically independent genes concerned with arginine synthesis, while $A_1$ and $A_2$ symbolize their corresponding normal alleles, the zygote nucleus will have the constitution $A_1/a_1$ $A_2/a_2$. If both pairs segregate at the first meiotic division and the mutant alleles are carried in the same end of the ascus, the eight spores will be as follows:

$$A_1A_2 \quad A_1A_2 \quad A_1A_2 \quad A_1A_2 \quad a_1a_2 \quad a_1a_2 \quad a_1a_2 \quad a_1a_2$$

or the equivalent of this with the entire order reversed. The fact that four spores received normal alleles of both genes shows that each of the remaining four

spores must carry the two mutant alleles. The same reasoning would of course apply if both gene pairs showed second division segregation and four spores grew normally on minimal medium. The above genetic diagnosis of double mutant spores can be confirmed by crossing strains derived from them to wild type. If the identification is correct, some of the asci from the cross will carry eight mutant spores, a result not possible if the mutant strain differed from wild type by a single gene. The genetic nonidentity of the two mutant alleles $a_1$ and $a_2$ can be confirmed by the heterocaryon test. If $a_1$ and $a_2$ strains of the same sex are grown together on a medium containing a small amount of arginine (the amount carried over in conidia is sufficient) hyphal fusion will occur giving rise to a mycelium containing two types of nuclei $a_1A_2$ and $A_1a_2$. If both $a_1$ and $a_2$ are recessive to their normal alleles the heterocaryon will be the physiological equivalent of a double heterozygote in a diploid and will grow in the absence of arginine. Because of the possibility of dominance of either or both mutant alleles, however, a failure of the heterocaryon to grow normally cannot be accepted as evidence of allelism of $a_1$ and $a_2$ (4).

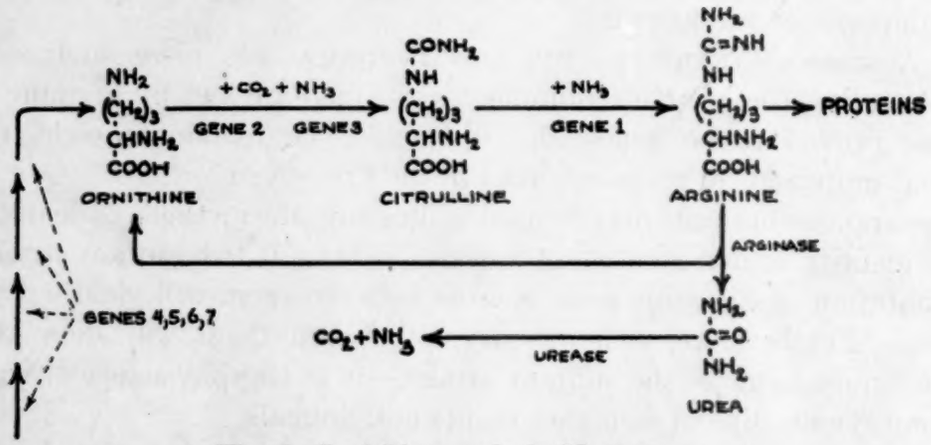


Fig. 3. Ornithine cycle in Neurospora.

In the analysis of "arginineless" mutants, Srb and Horowitz made use of these types of genetic tests. Characterization of the seven genetically distinct mutants in terms of their requirements for normal growth showed that one responded only to arginine, two grew normally in the presence of either arginine or citrulline, while the remaining four responded to arginine, citrulline, or ornithine. It is significant that for the conversion of citrulline to arginine which is most probably a single reaction, only one gene is known. Two genes are known for the conversion of ornithine to citrulline and there are good chemical reasons for supposing that this transformation involves two reactions with carbamino ornithine as an intermediate. The reactions by which plants synthesize ornithine from simpler constituents are not known. Four genes have been shown to be concerned with reactions leading to ornithine and on the basis of the one-gene-one-reaction concept this would imply at least four reactions in its synthesis, an implication that is certainly not unreasonable from a chemical standpoint.

In *Neurospora*, as in the mammalian liver, arginase is present and catalyzes the splitting of arginine into ornithine and urea. Unlike the animal which

excretes the urea, *Neurospora* further degrades it into carbon dioxide and ammonia under the influence of the enzyme urease. It is clear that the only reasonable interpretation of the combined genetic and biochemical data involves the assumption that in *Neurospora* there is an "ornithine cycle" (fig. 3) fundamentally similar to that postulated for the mammal by Krebs and Henseleit (29). The part played by genetics in the discovery of this in *Neurospora* is evident. Without the mutant strains studied by Srb and Horowitz it would have been difficult indeed to demonstrate. From the standpoint of comparative biochemistry, it is most significant that organisms as far apart as *Neurospora* and mammals should have systems of reactions involving arginine that are so strikingly similar. It is probable that the ornithine cycle constitutes a basic process in the metabolism of protoplasm in all organisms.

IV. Biosynthesis of tryptophane. A frequent type of mutant in *Neurospora* resulting from x-ray or ultraviolet treatment is one requiring an exogenous

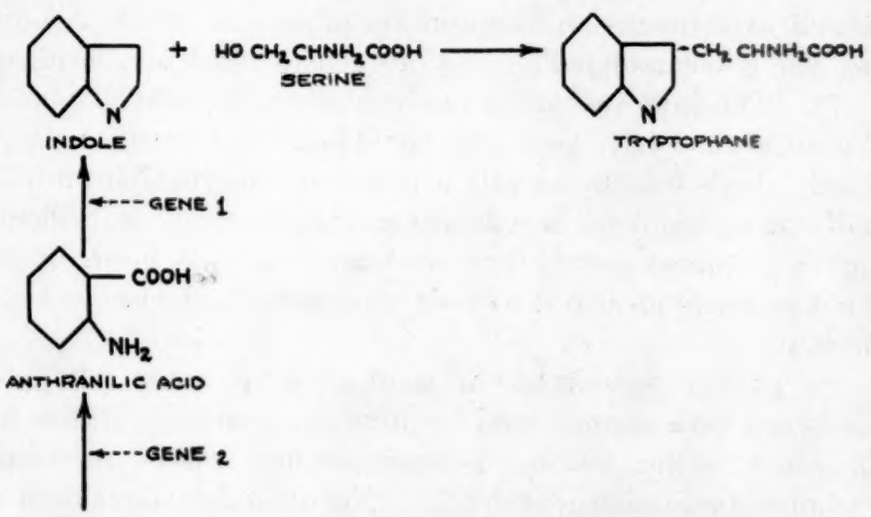


Fig. 4. Trytophane synthesis in Neurospora.

source of tryptophane or some related compound for normal growth. Among more than 30 occurrences of mutants of this category genetic tests show at least two to be distinct. Crosses between them give wild type recombinations and a heterocaryon resulting from their union shows substantially normal growth in the absence of tryptophane (61). Tatum and Bonner (60) have shown that for one of these mutants indole can replace tryptophane. In studying the uptake and utilization of indole, it was noted that the presence of serine in the medium markedly accelerates the disappearance of indole. The further observation was then made that following the uptake of serine and indole, tryptophane appears in the medium. The hypothesis that this is formed by condensation of indole and serine is supported by several lines of evidence (60). As a matter of fact, wild type strains are as capable of carrying out this reaction as the mutant strains used in the first experiments, but it is interesting to reflect that the experiments would in all probability not have been undertaken in the first instance had it not been for the mutant strain.

A second type of mutant can utilize either indole or anthranilic acid in place of tryptophane, while anthranilic acid cannot be used by the first one. This suggests anthranilic acid as a precursor of indole and this suggestion gains strong support from the finding that in the presence of a minimal amount of tryptophane or indole (for growth), the first mutant strain accumulates anthranilic acid and releases it into the medium. It has been isolated in pure form and identified chemically. Evidently the mutant gene of strain 1 is related to the reaction by which anthranilic acid is converted to indole. In mutant 2 the synthesis of anthranilic acid is blocked. These relations are shown diagrammatically in figure 4. It is interesting that no mutant has so far been found in which the condensation of indole and serine is blocked. There has been found a mutant type in which serine synthesis is interfered with in some way (Hungate, unpublished; 27) but detailed studies on this have not yet been reported.

These studies on tryptophaneless mutants are an example of how genetics can be of use in studying metabolic processes. It is almost exactly analogous to the classical experiments on alcaptonuria in man in which 2,5-dihydroxyphenylacetic acid is accumulated because of a genetic block preventing its further oxidation (17). This intermediate in the breakdown of phenylalanine and tyrosine would probably not have been discovered had it not been for alcaptonurics. In normal individuals it is apparently a transitory intermediate in metabolism and seldom if ever accumulates in sufficient amount to permit of its identification. Similarly in tryptophane metabolism, anthranilic acid is normally transitory and would not be easily identified without using genetic blocks to "isolate" it as a metabolic step.

V. Defects in the synthesis of other amino acids. In addition to mutants concerned with arginine and tryptophane synthesis, strains have been detected in which lysine, leucine, isoleucine-valine, valine, methionine, and proline are required for normal growth (27). Not all of these have been subjected to detailed study, however.

Doermann (15) has described a strain requiring an external source of lysine for normal growth. It is differentiated from the normal strain by a single gene which lies on the sex chromosome. An interesting property of this strain is that it is specifically inhibited by arginine. If the molar ratio of arginine to lysine exceeds one, regardless of the absolute amounts of either in the medium, growth is inhibited. This inhibition is removed by adding an excess of lysine regardless of the absolute amount of arginine present. No inhibiting effect of arginine on wild type strains has been observed at any concentration. The explanation of this phenomenon of specific inhibition is not obvious. It is possible, as Doermann suggests, that it may involve some mechanism in which competition plays a part.

Regnery (43) has studied a mutant strain, differing from wild type by a single gene change, which requires leucine for normal growth. Its growth responses to various possible leucine precursors have not, however, clearly indicated the mechanism by which this amino acid is normally synthesized in *Neurospora*.

One single-gene mutant strain has been found with a double growth factor

requirement (6). This strain requires a supplement of both isoleucine and valine in a ratio approximately 7:3. All attempts to find a single compound capable of restoring its growth rate to normal have been unsuccessful. At first sight it might appear that this case does not agree with the assumption that a single reaction should be modified by a change in a single gene. It is possible, however, to suppose that in the formation of these structurally similar amino acids, there is a common reaction. There might, for example, be a common step in the formation of their carbon skeletons, each of which has a branch at the beta position. Or it is conceivable that the amination of two homologous alpha-keto, beta-methyl acids might be catalyzed by a common enzyme. However, attempts to determine the nature of this common step, if there be one, and the structure of the natural precursors of valine and isoleucine have so far been unsuccessful; the final interpretation of the isoleucine-valine mutant must therefore await additional information.

VI. Biosynthesis of vitamins. It is of interest to note that no clear case has yet been reported of a plant requiring any vitamin other than those of the B-group (47). In the hundred or more induced mutant strains in *Neurospora*, it is likewise true that none requires vitamin C or any of the fat-soluble vitamins. It is possible that through some unknown systematic factor in the experimental procedure these have occurred but were discarded. This seems unlikely, however. The important question arises as to the rôles, if any, of the fat-soluble vitamins in plants. If they play no part in the plant's metabolic economy, why were the reactions leading to their synthesis retained during the long course of evolution? It seems more probable that they are of basic importance in ways not yet understood than that they are elaborated purely incidentally. It is most probable, for example, that the carotenoids of higher plants are of some unknown significance for, otherwise, why should they be invariably associated with chlorophyll (56)?

So far as the vitamins of the B-group are concerned, *Neurospora* mutant strains have been found each of which requires the following: thiamin (57, 58), pyridoxin (5, 53, 57), *p*-aminobenzoic acid, (59), pantothenic acid (27, 57), inositol (2), nicotinic acid (27, 57, 58), and choline (26). Omitting biotin which is required by all strains, this list includes all known B vitamins except riboflavin and folic acid. No fungus requiring riboflavin has been reported although many if not all synthesize it (57). Recently a mutant strain of *Neurospora* has been found in which riboflavin is required, but only under special circumstances (Mitchell and Houlahan, unpublished).

A mutant strain of *N. sitophila* requiring thiamin or thiazole is known to differ from the normal strain by a single gene (5). A second strain requiring thiamin is known in *N. crassa* and here the specific error in synthesis appears to lie in the reaction by which the thiazole and pyrimidine halves of the molecule are joined since the thiazole and pyrimidine halves supplied simultaneously do not promote growth (58). A pantothenic acid-requiring mutant strain of *N. crassa* appears to be unable to combine the pantoyl lactone and beta-alanine components of the pantothenic acid molecule, although this can be accomplished by certain other

organisms (57). If the moities are supplied separately or together as a mixture they are ineffective in promoting growth (57). An inositolless strain of *N. crassa* is known. No other compound is known which will promote its growth. Two genetically distinct nicotinicless strains are known (4), but studies on their biochemical characteristics have not yet been completed.

A strain of *N. sitophila* requiring pyridoxin has been reported (5) and studied in more detail by Stokes, Foster and Woodward (53). This is of particular interest in that at a pH of above 5.8 and in the presence of ammonia nitrogen, pyridoxin is synthesized in amounts sufficient to give approximately normal growth. Other cases are known in which specific syntheses are dependent on pH of the medium and on other cultural conditions (27, 57), one of them in arginine synthesis in *Neurospora* (55). The mechanism by which pyridoxin synthesis is dependent on pH is not known, but two possible interpretations have been suggested (57): (1) that the limiting reaction is not completely blocked and at the higher pH has a rate sufficient to cover the needs of the organism, and (2) that normally two pathways are open for certain steps in synthesis, one taken at a low pH range, the other at a higher pH, and that only the low pH route is blocked in the pyridoxinless strain. Another possibility is that a pH-sensitive threshold level of pyridoxin is needed and with the same absolute amount of pyridoxin this is exceeded at one pH but not at the other. In any case, there is no need whatever to assume a change in the gene with change of media.

That *p*-aminobenzoic acid is essential for the metabolism of *Neurospora* is shown by the occurrence of a mutant strain unable to grow normally in its absence (59). This strain, which differs from normal by a single gene, is able to use certain analogues of *p*-aminobenzoic acid, such as *p*-nitrobenzoic acid, but the activities of these are so low that they are probably not normal precursors of *p*-aminobenzoic acid. The introduction of nitrogen into this vitamin probably precedes ring closure in its biosynthesis.

Horowitz (27) has studied the interrelations of two genetically distinct strains both of which require choline or some related compound for normal growth. One of these is evidently blocked between a particular precursor of choline and choline itself, while the other is blocked prior to the precursor. This is judged to be so because in the presence of minimal amounts of choline the first mutant accumulates a substance which is not choline but which will replace choline in promoting growth of the second mutant (27). Studies on the nature of the precursor have not yet been completed.

VII. Miscellaneous syntheses. Loring and Pierce (35) have studied a monogenic mutant strain of *N. crassa* which is unable to grow normally unless supplied with a pyrimidine or a pyrimidine derivative. Uracil as a free base will promote growth but has only 1/60 to 1/10 the activity of its nucleoside. Cytosine is itself inactive but cytidine and cytidylic acid are as active in promoting growth as their uracil analogues. These results suggest that cytosine is not a normal precursor of either its own nucleoside or nucleotide, or of uracil (35).

The first step in the utilization of nitrate by a plant apparently involves its reduction to nitrite. This is then further reduced and may subsequently be

incorporated in various organic compounds. A strain differing from wild type by a single gene is known in which the reduction of nitrate to nitrite is blocked. Nitrite, ammonium, or amino nitrogen permits normal growth of this strain (27).

Horowitz (27) has studied two mutants in which fat metabolism is altered in some way. Wild-type strains of *Neurospora* are able to utilize fats and certain fatty acids as sources of carbon and energy. One of the mutant strains is entirely unable to utilize any of a number of fatty acids tested, while the other is able to utilize saturated fatty acids but not their unsaturated analogues.

In addition to the types of mutants already considered, a second category is found. This includes those which grow on complete medium (containing yeast and malt extracts) and not on minimal, but which so far have not been induced to grow in the presence of any known compound. These mutants in which unknown syntheses are blocked are fundamentally no different from those already mentioned. On further study some of them will undoubtedly prove to require known substances that have not yet been tested or have not been tested under the proper conditions. There may be a final residue which requires compounds not at present suspected of having biological significance.

VIII. Mutant strains and bioassays. A strain in which a particular reaction is genetically blocked is, in general, able to use any compound in the reaction chain that normally arises subsequent to the block—if the compound is able to enter the cell from the outside. If the ultimate reaction in the formation of a given end product is blocked, the strain will respond specifically to the final compound. Thus the argininеless mutant which is unable to convert citrulline to arginine responds specifically to arginine (55). On the other hand, a strain unable to synthesize ornithine will be expected to respond to arginine, citrulline, ornithine, and possibly to precursors of ornithine.

The quantitative growth response of a mutant strain to compounds active in promoting its growth often provides a basis for a useful method of bioassaying for these compounds. As is well known, such biological assays often have high sensitivity and specificity and can be used on complex mixtures. It is obvious that the specificity will vary depending on the genetic constitution. Thus, in the arginine series, any of three or more specificities are possible. If one were interested in arginine alone, the strain responding only to arginine would be selected. Usually with naturally occurring organisms the choice as to specificity is limited and this is often true with artificially produced mutant strains as well. No strain of *Neurospora* has been produced, for example, that specifically requires tryptophane; all are sensitive to indole as well.

Bioassay methods using *Neurospora* mutant strains have been developed for *p*-aminobenzoic acid (62), pyridoxin (54), choline (24, 26, 36), leucine (43, 46), and inositol (2).

IX. Mechanism of gene action. The types of mutant strains so far found in *Neurospora* probably make up a reasonable sample in indicating the kinds of reactions subject to genetic control. If to these are added those reactions known to be gene controlled in other organisms (3), there can be no slightest doubt but that the chemical versatility of the gene is great. In fact, a convincing argument can be made that if suitable experimental conditions were possible and if one

looked sufficiently long, mutation could be detected in a gene concerned with any predetermined enzymatically catalyzed reaction. This has almost been achieved for certain sets of reactions in *Neurospora* and the possibilities have by no means been exhausted.

Assuming that it is true that every biochemical reaction has a specific gene directing its course, how is this control achieved? Unfortunately, we can only speculate as to the final answer to this question. Such speculation is not entirely without basis, however, and is perhaps justified by the importance of the question.

Genes are thought from various lines of evidence (3, 21, 39, 41, 48) to be composed of nucleoproteins or at least to contain nucleoproteins as essential components. They have the ability to duplicate themselves which of course they do once every cell division. The manner in which this self-duplication is brought about is one of biology's unsolved problems, but it is thought to involve a kind of model-copy mechanism by which the gene directs the putting together of the component parts of daughter genes (8, 22, 23, 66). If this is the mechanism and genes contain protein components, gene reproduction is a special case of protein synthesis. Since many cases are known in which the specificities of antigens and enzymes appear to bear a direct relation to gene specificities (3, 66), it seems reasonable to suppose that the gene's primary and possibly sole function is in directing the final configurations of protein molecules.

Assuming that each specific protein of the organism has its unique configuration copied from that of a gene, it should follow that every enzyme whose specificity depends on a protein should be subject to modification or inactivation through gene mutation. This would, of course, mean that the reaction normally catalyzed by the enzyme in question would either have its rate or products modified or be blocked entirely.

Such a view does not mean that genes directly "make" proteins. Regardless of precisely how proteins are synthesized, and from what component parts, these parts must themselves be synthesized by reactions which are enzymatically catalyzed and which in turn depend on the functioning of many genes. Thus in the synthesis of a single protein molecule, probably at least several hundred different genes contribute. But the final molecule corresponds to only one of them and this is the gene we visualize as being in primary control.

It is by no means an easy matter in all cases of gene change to determine what particular chemical reaction is modified—or even that only a single one is modified. Even after a given reaction is specified as the one modified in a particular mutant strain, the mutation may effect it only indirectly. In the case of the mutant strain of *Neurospora* that cannot combine the two components of the pantothenic acid molecule, we infer that a specific enzyme concerned with this reaction is inactivated. But it is possible, if the enzyme concerned depends for its activity on a prosthetic group or coenzyme, that the enzyme is inactive either because of an inactive protein configuration (primary gene control) or because of absence of coenzyme or prosthetic group (secondary control). Since in this particular case the enzyme concerned is not known, the distinction is not at present easily made on the basis of experimental data.

X. Evolutionary implications. From the work on *Neurospora* the genetic basis of the evolutionary specialization of organisms with regard to nutritional requirements becomes clear. Starting with a completely autotrophic organism it is easy to see how by gene mutation it could become progressively simpler so far as its synthetic abilities are concerned and correspondingly more exacting in its nutritional requirements. That just this kind of specialization has occurred in many groups of organisms is clear from the work of Lwoff (37), Knight (28), Schopfer (47) and others. With the evolutionary development of parasitism, for example, it is to be expected that, since many compounds can be obtained from the host, the ability of the parasite to synthesize these will in many instances be of no selective advantage. Since loss by mutations are known to be more frequent than the reverse it is statistically inevitable that eventually a gene that confers no selective advantage will become inactive or be lost. Among the fungi, however, Schopfer (47) points out that the ability of parasitic forms to synthesize vitamins of the B group is sometimes retained in spite of the parasitic habit. Presumably the ability of the organism to carry out these syntheses is of some advantage that is not immediately evident.

From a genetic standpoint specialization through loss of synthetic ability would be expected to be difficult but not impossible to reverse. Consider, for example, the case of thiamin synthesis. If an organism able to synthesize this vitamin and incorporate it into its enzyme system is able to obtain free thiamin in abundance in the medium in which it grows, a mutation resulting in the loss of ability to synthesize it will not be selected against and will be expected largely to replace the original form as a result of mutation pressure (greater rate of mutation from the active form of the gene to an inactive form than the reverse). If the ability to combine thiazole and pyrimidine is lost in this way it is of no advantage to the organism to synthesize these components unless they serve some independent need that is not satisfied by the exogenous thiamin. The ability of the organism to make these will therefore tend also to be lost through mutation. If this happens for both components, at least three mutations will have become established. Even if each is reversible, the probability of all three mutating back to normal simultaneously becomes small. If any one of these genes becomes completely lost, restoration of the synthesis through mutation becomes even more difficult.

If evolutionary loss of synthetic ability is a process so difficult of reversal, it is fair to ask how elaborate systems of reactions leading to the final synthesis of a desired end product ever arose in the first place. If this depended on the simultaneous mutation of genes controlling all steps in the synthesis it seems almost inconceivable that it could have occurred, particularly if totally new types of genes had to arise from existing ones through mutation. If each intermediate compound in the system were independently useful to the organism the series could evolve from the beginning and progress step by step toward the final product, with each successive step having a selective advantage over the preceding one. It seems unlikely, however, that the rather severe restrictions imposed by such a system would be met. Horowitz (25) has recently made the suggestion that if we accept the thesis of Oparin (42) and others that the first

self-duplicating units of life reproduced by taking up preformed relatively complex organic molecules from a medium in which they had spontaneously accumulated, it is tenable to suppose that the evolution of systems of reactions took place backward from the final product. In this way each successive step in the process would make the organism less dependent on organic molecules and would have a corresponding selective advantage. The evolution of a completely autotrophic organism seems at least conceivable on such a basis. An important corollary of this hypothesis of the backward evolution of syntheses is that it is unnecessary to assume that all lines of descent trace back through completely autotrophic ancestors.

As Horowitz points out, many present day parasites live in environments essentially similar to the hypothetical world of free organic molecules in which Oparin assumes life to have originated. It is conceivable that such parasites might under certain conditions reverse their evolution trends and again progress in the direction of autotrophism. However, while this possibility must not be excluded, the probability of its happening must be negligible as compared with the probability of specialization through loss of synthetic ability.

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# RELATIVE NUTRITIVE VALUES OF ANIMAL AND VEGETABLE FATS

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The nutritive value of fats has engaged the attention of scientific investigators for many years. More recently, as a result of the war situation, the question of fat supply and best utilization of what is available has resulted in considerable attention being focussed on this problem. Certain fats, notably butter, natural vegetable oils and their hydrogenated derivatives, and the margarines have assumed especial prominence in this connection.

The nutritive value of fats can be considered from several points of view. It is obvious that any fat, in order to serve as food, must be capable of both assimilation and then utilization in metabolism. This involves digestion and absorption. One may therefore consider first of all the extent to which various edible fats are digested and absorbed. Fats are important sources of energy to the body and therefore may be compared with respect to their yield of calories. To a very large extent questions under this heading would be answered by the available data pertaining to the digestion and utilization of different fats. Edible fats also serve certain non-caloric functions. Their value in nutrition can vary according to their composition and content of essential fatty acids, notably, linoleic acid. Edible fats are known to vary in their content of fat-soluble vitamins and this can serve as another basis for estimating their value in nutrition. There are claims in the literature to the effect that certain fats contain unknown fat-soluble dietary essentials remaining to be isolated and identified. Fats, therefore, may be rated according to their content of such unknown factors. Perhaps the most important basis upon which to rest any nutritive comparisons of fats is their value for promoting certain biological functions, notably, those of growth, pregnancy and lactation. The most recent work relating to our theme has centered around a study of this particular topic.

It is pertinent to indicate the limitations of the present discussion. It will not consider: (a) the mechanisms and theories of digestion and absorption, (b) the intermediary metabolism of fats, and (c) clinical phases of the topic. For discussion of these subjects the reader is referred to several important reviews, notably that by Anderson and Williams (1), the article by Bloor (2) and that by Burr and Barnes (3), all of which have appeared in this Journal. Another review of interest is a publication of the Council on Foods and Nutrition of the American Medical Association (4). A short review of the most recent work has also appeared in Nutrition Reviews (5). In addition to the foregoing the reader would do well to consult the chapters on fat metabolism that have appeared in various volumes of Annual Reviews of Biochemistry the first of which was published in 1932.

DIGESTION AND UTILIZATION OF FATS. Studies of the digestion and utilization of various fats have been conducted on several species. The observations

of Langworthy and associates (6) made on students or laboratory assistants are noteworthy. The digestibility of 23 animal, 34 vegetable and 6 hydrogenated fats was studied by feeding amounts ranging from 50 to 150 grams. The fats were incorporated into a corn starch pudding which was fed as part of a simple standard basal ration. Analyses were made for fecal fat. No pronounced differences were noted in the digestibility of the fats studied. There was a suggestion that the thoroughness of digestion was inversely proportional to the melting point in the case of the hydrogenated fats; those hydrogenated to a definite melting point, and those made by mixing soft and hard fats showed no definite differences in thoroughness of digestion. In only two cases was a laxative effect noted, these being cacao butter and goose fat. The illustrative digestibility coefficients for certain commonly used fats are the following: butter fat 97 per cent, beef kidney fat 88 per cent, cocoanut oil 97.9 per cent, cocobutter 95.9 per cent, corn oil 96.8 per cent, and soy oil 97.5 per cent. In an earlier report Holmes and Deuel (7) gave data on digestibility coefficients for corn oil, cottonseed oil and peanut oil that had been hardened to varying degrees by hydrogenation. For hardened cottonseed oil with melting points 35°, 38.6°, and 46°, the respective digestibility coefficients were 96.8, 95.5 and 94.9 per cent. Similar data for hydrogenated peanut oil with melting points 37°, 39°, 43°, 50° and 52.4°, were, respectively, 98.1, 95.9, 96.6, 92.0 and 79.0 per cent. Corn oil hardened by hydrogenation so as to have melting points of 33°, 43° and 50° had 94.7, 95.4 and 88.5 per cent as their respective coefficients. In general, digestibility decreases as the melting point increases. All the fats melting below 46° were well utilized. The same authors (8) studied the digestibility of cod-liver, java-almond, tea-seed and watermelon seed oils, deer-fat and some blended hydrogenated fats. The blended fats with different melting points prepared from corn, cottonseed and peanut oil showed coefficients of digestibility ranging from 91.5 to 97.4 per cent; a blended cottonseed fat that melted at 50° gave a coefficient of 97.0 per cent. The coefficients for cod-liver, java-almond, tea-seed, watermelon seed oils and deer fat decreased in the order named from 97.7 to 81.7 per cent.

Some observations have been made of the digestibility of fats of different tissue origins. Holmes (9) tested fats obtained from different parts of the body of cattle but not from the same animal. Two rather unusual items were tested, namely, hard-palate fat and ox-tail fat. Tests were also made of butter and cream for purposes of comparison. The digestibility of brisket fat, ox-tail fat, butter and cream proved to be 97 per cent; the value for hard-palate fat, kidney fat and ox-marrow fat was 94 per cent; for oleo oil and oleostearin the values were 97 and 80 per cent, respectively. An interesting observation was that ox-marrow fat caused distressing disturbances in all subjects when the smallest test dose, namely, 59 grams, was ingested daily. The other fats were given in amounts ranging from 59 to 100 grams daily and produced no abnormal physiological reaction.

Corn oil and cottonseed oil have been tested in dogs (10) and found to be 99 per cent digestible.

Holt and associates (11) determined the digestibility coefficients in normal infants and obtained the following results: corn oil 96.9, soy bean oil 93.7, cocoanut oil 88.7, butter 88.9 and a mixture of butter and corn oil 96.6 per cent. It will be noticed that these values are not greatly different from those reported by Langworthy, Holmes and Deuel. In an estimation of the value of various fats for premature infants Tidwell et al. (12) noted that in such infants olive oil and soy bean oil are better absorbed than butter fat.

Steenbock, Irwin and Weber (13) studied the percentage of fat absorbed from the alimentary canal of rats at 2, 4, 6, 8 and 12 hours after feeding definite quantities of fat. It was found that: (a) partially hydrogenated vegetable oils, as sold commercially for home and bakers' use, were absorbed as rapidly as lard or corn oil; and (b) that butter oil, halibut liver and cod-liver oil were absorbed uniformly at a more rapid rate than corn oil, lard, or the partially hydrogenated fats. Eleven other samples of fat tested after a 4-hour absorption period could be arranged in the following descending order of their percentage absorption: linseed oil, olive oil, whale oil, soy bean oil, peanut oil, rancid lard, cottonseed oil, cocobutter, cocoanut oil, palm oil and oleo stock. The authors make the statement that "although the difference between any of these fats and the one immediately preceding or following in the list may not be significant, certainly there were very real differences in the absorption rates of those widely separated." Studies of this type using the rat as the experimental animal have also been made by Hoagland and Snider (14). Under the conditions of their experiments there was no consistent relationship between the melting points of the fats and their digestibility coefficients. When the diets contained 5 per cent of fat, the following digestibility coefficients were obtained: cocoanut oil 98.9, soybean oil 98.5, corn oil 97.5, butter fat 88.3, mutton tallow 74.6, oleo stock 74, and cacao butter 63.3 per cent. These results should be compared with those obtained in experiments where the diets contained 15 per cent of fat. In a few instances this feeding of higher level of fat improved the coefficients somewhat, notably those of the fats which rated low when only 5 per cent of fat was fed. The results were: soy bean oil 98.3, corn oil 98.3, cocoanut oil 96.5, butter fat 90.7, oleo stock 86.7, mutton tallow 84.8, and cacao butter 81.6 per cent. In experiments with men, made as part of a study of whole wheat and white breads, Sealock, Basinski and Murlin (15) observed a high digestibility of the fats present in the mixed diets, the digestibilities ranging from 95.7 to 97.4 per cent.

NON-CALORIC FUNCTIONS OF FAT. *Fats as sources of essential fatty acids.* Considerable information on this subject has accumulated since the pioneer observations of Burr and Burr (16). Since it was reviewed in 1943 by Burr and Barnes the topic does not need extended discussion here. Let it suffice to quote a summary statement from the Burr and Barnes review (3, p. 261):

> Those acids which have been reported to have some curative effect upon fat deficient rats are linoleic, linolenic, arachidonic, decosahexanoic and hexahydroxystearic. In addition, linoleyl alcohol is useful. All other fatty acids and isomers of the above compounds have been reported negative. Linoleic, arachidonic and linolenic acids are the only ones extensively worked with. Quantitative comparisons of their growth effects,

made in different laboratories, are not in close agreement but it is clear that linoleic and arachidonic are much alike while linolenic is inferior. This acid produces some growth but does not cure the skin, reduce water consumption to normal or improve lactation (Burr, 1942; Quackenbush et al., 1942) (Burr, 1942, Fed Proc. **1**: 224; Quackenbush, Kummarow and Steenbock, 1942, J. Nutrition **24**: 213). Decosahexoic acid and the mixed esters of cod liver oil react in a similar way and it can be said that linoleic and arachidonic acids are highly specific in their effects upon the skin.

The foregoing quotation of course suggests that the distribution of these particular fatty acids in vegetable and animal fats should be ascertained.

One cannot, however, dispose of this problem merely by considering the amounts of these fatty acids present in various fats, because there are observations suggesting that essential fatty acid, pyridoxine, pantothenic acid and perhaps even other factors may in some way be related to the prevention and cure of "acrodynia" in rats. This is illustrated by the striking curative effect of linoleic acid on the acrodynia-like disease produced in rats on fat-low diets observed by Quackenbush, Platz and Steenbock (17) who wrote as follows: "Complete healing of either the acute or chronic form was obtained by the administration of peanut oil or wheat germ oil. . . . One-half drop of ethyl linoleate per day cured the dermatitis completely." In another paper (18) this same group of workers confirmed numerous other investigators who had called attention to members of the B-complex group of vitamins being involved in the production and prevention of this experimental acrodynia.

Linoleic acid when fed as the ethyl ester at a subcurative level, viz., 5 mg. daily, produced an effect similar to that obtained with pyridoxine. When fed at higher levels, viz., 10 mg. or more, the acrodynia was cured completely. . . . Pyridoxine together with pantothenic acid produced a greater improvement than was obtained with pyridoxine alone, but less than linoleic ester or a commercially available rice bran concentrate. . . . Pantothenic acid alone did not even alleviate the symptoms. . . . The three compounds together cured the acrodynia but did not cure completely the scaly condition of the tail and hind-paws.

These authors considered their results to indicate that an additional factor is involved.

*Fats as sources of known vitamins.* It has long been appreciated that fats vary with respect to their content of the known fat-soluble vitamins. Numerous tables of food composition have been published which may be consulted for detailed data on this subject. The discussion here will be limited to a few special points of interest.

With respect to vitamin A and its provitamin carotene, the latter and its biologically effective carotenoid relatives constitute the form to be found in plant tissues such as, for example, the leaves of spinach, greens, and alfalfa. The vitamin A potency of butter is due partly to carotene and partly to vitamin A itself. Potent intracellular fats of animal origin, cod liver oil for example, contain vitamin A as such. The fats found in the fat depots of the animal body rate so low in vitamin A content as to constitute an insignificant source of this vitamin for human nutrition. Similarly, the fat depots in plants, oily seeds for example, are, broadly speaking, practically devoid of vitamin A potency. These

are the important commercial sources of such oils as palm oil, cottonseed oil, olive oil, etc. These oils are found in the kernels of the respective seeds, and are to be distinguished from the oils that can be extracted from the outer covering or cortex of the seeds, some of which have been found to be good sources of carotene (19). Rosedale (20) has reported a rather high vitamin A potency for the red palm oil of Malaya, *Eloeis guineensis*. The Indian Research Fund Association has suggested that through more extensive cultivation in India of the palm from which this oil is obtained and education of the Indian population concerning the value of this oil as a food much could be done to improve the vitamin A intake of the people of that subcontinent (21). Buckley (22) has determined biologically the carotene content of this red palm oil as affected by degree of ripeness and noted some differences; oil from the ripe fruit contained 1900 international units of vitamin A per gram, that from over-ripe fruit 1600 I.U./gram, and the unripe fruit only 600 I.U./gram. Palatability, and concentration of the carotene could be increased by removal of the solid constituents that settle from the oil which is normally liquid at Malayan temperature. The Indian Research Fund Association (21) made a study of the possibilities for use in India, of this red palm oil. The palm *Eloeis guineensis* is native to West Africa. By 1937 it had been introduced into Malaya and Burma but not into India. A clinical trial showed that a suitable emulsion was just as effective as cod liver oil in severe cases of keratomalacia in Madras. It was roughly calculated that in India a given amount of vitamin A potency as red palm oil would cost about one-third of the cost of the same amount of cod liver oil. The oil could be used medicinally or as a culinary fat or, when mixed with cocoanut oil or hydrogenated fat, as a kind of butter or margarine. De (23) fried chipped potatoes in mixtures of red palm oil and various other vegetable oils at temperatures of 100 to 140°C., and found the resulting product to contain substantial amounts of carotene and to be quite palatable. This investigator (24) has also reported on the carotene content of about 80 vegetable foods eaten in India. Observations of the sort just cited could doubtless be made with respect to still other valuable sources of carotene available in various parts of the world, particularly in the tropics, and as yet not used as human food to the extent that their nutritive values warrant. The greater tendency of fats to turn rancid in the tropics probably means that rich sources of carotene constitute the most practical ways for providing vitamin A to the masses living in the tropic zone. This need not be the case for all of the inhabitants in such areas in view of recent researches aimed at providing American troops stationed in the tropics with butter and related fats that have been stabilized, so to speak, so as to be capable of storage for considerable periods without deterioration of any kind. Presumably such newly developed products can be made available in tropical countries, but the amounts provided would doubtless furnish but a small part of the total need of the population. In arctic regions, on the other hand, fish oils play a similar rôle.

In the temperate zone, particularly in the United States and certain other countries that have a well developed dairy industry, butter is one of the most important sources of vitamin A. In recent years many margarines made largely

if not entirely from vegetable oils have been developed and used as butter substitutes. Most margarines in the United States are now fortified with vitamin A, and a standard for such a product has been established by the Food and Drug Administration of the federal government (25). This standard requires that fortified oleomargarine contain not less than 9000 I.U. of vitamin A per pound. This value was selected after consideration of the data on the vitamin A content of butter that were available at the time the standard was being considered. It is known that the vitamin A value of butter depends upon the feed of the cow; the values are low in the winter season and high when the animals are given access to green pasturage. The most recently obtained data are well illustrated by those published by Berl and Peterson (26) who analysed numerous samples of Wisconsin butters: "March butters averaged 9500 I.U./lb. The butters of July and September averaged about 18,000 I.U./lb. The January butters were slightly better than the March butters and contained about 10,500 I.U./lb. In the summer butters, about 75 per cent of the total natural butter pigment was found to be carotene; in the winter butters 60 to 65 per cent of the pigment was carotene."

With respect to vitamin D, the analyses published by Wilkinson (27) indicate that butter contains from 50 to 500 I.U./lb. The margarines are practically devoid of vitamin D unless fish liver oils or their concentrates are the sources of vitamin A used in the fortification. In England both vitamins A and D are added to margarines. According to the compilations given in Miscellaneous Circular 275 of the United States Department of Agriculture (28) natural foods which contain vitamin D are of animal origin. Fish which contain much body oil, such as salmon, herring and sardines, are the richest natural sources; eggs come next, then milk fat; meat products contain some vitamin D.

The richest natural sources of vitamin E are the oils contained in plant embryos. Wheat germ oil is the prime example here. The margarines can therefore contain appreciable amounts of vitamin E depending upon the formula used. Butter has been shown to be low in content of this factor, but the amount present in whole milk has been shown to be sufficient to meet the needs for reproduction in dogs and rats. The significance of vitamin E in human nutrition has not been satisfactorily evaluated. Because of this fact, nutritional claims for vitamin E are as yet not recognized by the Councils of the American Medical Association and by the Food and Drug Administration of the federal government.

With respect to vitamin K little can be said. The amount of this factor in butter has not been determined accurately, but various considerations lead to the conclusion that the amount present must be small. The seeds of plants contain less than do the leafy parts of the plant. This suggests that the margarines should contain only small amounts. In view of the fact that the intestinal bacteria can synthesize all of the vitamin K needed by the body (at least that of adults), any slight differences which plant and animal fats may show in vitamin K content are probably of no nutritional significance.

*Fats as sources of unidentified dietary essentials.* The possibility that unknown dietary essentials are present in certain fats is suggested by the work of Wulzen

and associates which is interpreted to mean that cream contains an unknown factor needed by the guinea pig to prevent a marked stiffness and final rigidity of the wrists. The pioneer observations were made in experiments where planarian worms were fed various tissues of rats and guinea pigs that were deficient in vitamins A or B-complex or C (29). van Wagtendonk and Wulzen (30) finally announced the isolation of a curative "anti-stiffness" substance in crystalline form from raw cream, prepared by distilling fatty acids with steam and separating the active agent in the distillate from acidic compounds; final crystallization involved the preparation of a mercury iodide complex derivative formed with Girard's reagent. The vitamin recovered from this crystalline derivative was a pale yellow oil with ten million times the activity of the cream from which it was prepared. It was claimed that the vitamin is a carbonyl compound with a molecular weight of about 200. A dose of 0.1 microgram per day administered daily for five days sufficed to cure a guinea pig of the stiffness of the wrist joints. The symptoms exhibited by guinea pigs deficient in this "anti-stiffness" factor prove to be similar in some respects to those of lack of alpha-tocopherol and ascorbic acid, and one may raise the question whether the amounts of these dietary essentials in the experimental diet were adequate. In a later paper (31) it is shown, by a comparative study of the creatine excretion of animals on a diet deficient in the new dietary essential and in vitamin E, that these two factors are not the same: "A deficiency of the anti-stiffness factor produces in guinea pigs a characteristic wrist-stiffness but has no significant effect upon the creatine excretion."

A characteristic feature of the wrist-stiffness disease is the deposit of large amounts of calcium, mainly in the form of calcium triphosphate in connection with almost any body tissue, "conspicuous locations being muscle, subcutaneous tissue, aorta, liver and kidneys. This abnormal deposition of calcium phosphate suggested a derangement of the phosphorus metabolism." For this reason a study was made of the distribution of the acid-soluble phosphorus in the liver and kidneys of normal and deficient guinea pigs. The authors stated that "the interpretation of the changes in the distribution of the acid-soluble phosphorus in the liver and kidneys occurring in the course of the deficiency is as yet difficult." It was suggested that possibly "changes in the adenosine triphosphate and the adenosine diphosphate are of prominence during the deficiency, perhaps involving the whole metabolism of the purines." Further studies (32) revealed that along with these changes in the distribution of acid-soluble phosphorus there is a decrease in the alkaline serum phosphatase. Ascorbic acid in sufficient amounts to prevent scurvy did not prevent this drop in serum phosphatase level. On the other hand, "continuous administration of the anti-stiffness factor did prevent the low level."

With respect to the special theme of the present review it is pertinent to point out that the distribution of this factor among many fats has not been reported. Wheat germ oil and cod liver oil were reported to be ineffective. In many of the experiments the fat soluble vitamins were administered as solutions in cottonseed oil, but this cannot be interpreted to mean that the factor is absent from

cottonseed oil because the amount of the oil administered as a carrier of the fat soluble vitamins was undoubtedly small; it could be present but in an amount too small relatively to be significant in the experiment.

EFFECT ON THE REQUIREMENT OF OTHER NUTRIENTS. The "vitamin $B_1$ sparing action" of diets high in fat is one illustration of the way fats can influence the requirement of other nutrients. The early observations of this phenomenon by Evans and Lepkovsky (33, 34) were made on rats fed diets containing as the fat component either lard, cottonseed oil (Wesson), partially hydrogenated cottonseed oil (Crisco), butter, corn oil, cocoanut oil and walnut oil. The authors stated (34) that "all natural fats studied by us have shown the sparing action on the antineuritic vitamin B." It will be noted that the list just cited includes both animal and vegetable fats. The observations of Arnold and Elvehjem (35) were made on dogs fed diets in which the fat components of interest were autoclaved lard and cottonseed oil, one an animal fat, the other a vegetable oil. Stirn, Arnold and Elvehjem (36) experimented with growing rats and used butter fat, cocoanut oil, cottonseed oil, olive oil, peanut oil, and a group of autoclaved natural fats which included lard, peanut oil, cod liver oil and cottonseed oil; synthetic tricaproin and triacetin were also tested. All of the fats and oils showed this vitamin sparing action; no marked differences among them were noted. To this list of fats should be added certain other substances studied by Salmon and Goodman (37), namely, pecan oil and numerous synthetic esters of single fatty acids. These workers considered that cocoanut fat was the most effective natural fat tested in high fat diets with respect to effect in increasing the rate of growth and decreasing the incidence of beriberi in rats. "The effectiveness of esters of single fatty acids in alleviating the symptoms of vitamin B deficiency in rats depended upon the length of the carbon chain of the fatty acid. The effectiveness was maximum at the 8-carbon acid and decreased in each direction from this point." Much of the early work on this problem was complicated by the observation that the amount of autoclaved yeast used in the diet was a factor; also, the importance of essential fatty acid was not always realized, and therefore lack of this factor may have played a rôle. In the papers cited in this review the interested reader will find references to further literature dealing with these various points.

It is possible that high levels of fat in the diet increase the requirement for riboflavin. Mannering, Lipton and Elvehjem (38) fed groups of growing rats diets in which lard was isodynamically substituted for dextrin at two levels to give rations the total fat contents of which were 25 per cent and 40 per cent, respectively. Different groups of young rats subsisted on these rations supplemented with 3 micrograms and 6 micrograms of riboflavin. Increasing the fat level in a riboflavin-low ration adversely affected the growth of the animals; the administration of adequate amounts of riboflavin completely corrected the deficiency. The authors discussed possible explanations of this phenomenon: "Increasing the fat level in the diet may alter the intestinal flora so that less than normal bacterial synthesis, or more than normal bacterial destruction of riboflavin occurs, or riboflavin may be directly concerned with fat metabolism

or phosphorylation." It should be noted that only lard was used in these particular experiments. Presumably the Wisconsin workers have made additional unpublished observations with still other fats, for in a review article by the Council on Foods and Nutrition of the American Medical Association (4) this study is referred to and the following statement made: "In the rat, high levels of fat appear to increase the riboflavin requirement, but again all fats function equally." Observations on puppies, on the other hand, do not support such a conclusion. Potter, Axelrod and Elvehjem (39) reported the daily riboflavin requirement of the growing dog to be from 60 to 100 micrograms per kilogram bodyweight, and that the isocaloric substitution of lard for sucrose did not increase it. In these experiments the high carbohydrate diet contained 66 per cent sucrose, 8 per cent cottonseed oil and 3 per cent cod liver oil; the high fat diet was made by isocalorically replacing a portion of this sucrose with lard. It seems evident that further work is needed on this problem before a general relationship between level of fat in the diet and riboflavin requirement can be regarded as demonstrated.

Some reference should be made to what might be called the pyridoxine-sparing action of fats. In the early literature on vitamin $B_6$ one can find many instances where investigators found it more difficult to produce the typical picture of pyridoxine deficiency when the diet contained much fat; the feeding of a low-fat ration proved to be an important factor in such experiments. The work of Quackenbush et al. (18), already discussed, was interpreted in favor of the view that linoleic acid, pyridoxine, pantothenic acid and some additional factor are all involved in the cure of rat "acrodynia". It seems probable, therefore, that the rôle played here by any particular natural fat, whether of plant or animal origin, is related fundamentally to its content of essential fatty acid, particularly linoleic acid. It also seems evident that further studies of these relationships between essential fatty acid and certain B-complex vitamins are needed.

Choline is now known to be an important dietary factor. One of the significant features of choline deficiency in animals is fatty liver. This suggests that in any consideration of the relative nutritive values of plant and animal fats, possible relations to choline requirement should receive attention. The production of this particular form of fatty liver, however, is apparently related to a need for choline to assist in removing fat from the liver to fatty depots; to the extent that natural fats furnish phospholipids containing choline, such fats contribute something in meeting this need. It becomes of interest in this connection, therefore, to consider the distribution of choline in plant and animal products. According to Engel (40), "animal organs, egg yolk and nervous tissue were found to be considerably better sources of choline than any of the plant material examined. Green leafy plant material compared favorably with muscle tissue. Good sources . . . were found to be green leafy and leguminous vegetables, seed oil meals and grain germs. Seed oil meals were found to be equal or superior to the whole seeds . . . skim milk powder contained nearly a third more choline than whole milk powder." Careful examination of his data reveals that lard, cod liver oil, butter, hydrogenated cocoanut oil, oleomargarine and refined corn

oil, refined soybean oil all contained extremely small amounts of choline, actually less than 0.05 mgm./100 grams of fresh material. The significance of this figure is made evident by comparison with the following illustrative ones: beef liver 630, beef roundsteak 95, rolled oats 151, white flour 52, whole milk powder 107, fresh milk 14.7, potatoes 106. It is clear that many foods, cereals and skim milk for example, which in themselves are quite low in fat contribute most of the choline present in the diet along with other lipotropic substances. In the refining of natural fats phospholipids are almost entirely removed, and there are no significant differences between plant and animal fats in this respect.

Interesting observations relating fat to the retention and utilization of the galactose found in ingested milk sugar have been reported by Schantz et al. (41). Rats fed a diet of mineralized whole milk made efficient use of all the lactose as shown by the presence of only a trace of reducing substances in the urine and the absence of reducing substances from the feces. On the other hand, when skim milk was used instead of whole milk, after a few days the urine was found to contain appreciable amounts of reducing substances and the presence of significant amounts of galactose was definitely proven. Experiments showed that the 3 to 4 per cent of fat naturally present in the milk is necessary if the animal is to make complete utilization of the galactose. The milk fat could be replaced by other fats such as lard, corn oil, linseed oil, cocoanut oil, hydrogenated cocoanut oil, triolein (synthetic), tripalmitin (synthetic) and oleic acid. The following substances were not effective in this respect: tricaproin (synthetic), sodium lactate, sodium butyrate, beta-hydroxybutyric acid and choline. The list of substances tested was extended by Schantz and Krewson (42).

The triglycerides of caproic, caprylic, capric and lauric acids were synthesized and then homogenized into fresh skim milk at a level of 4 per cent. Within a test period of ten days caproin, caprylin and caprin were not effective, but laurin was. These same animals that were losing galactose via the urine when fed these ineffective fats, began within 3 or 4 days to retain the galactose when corn oil replaced these fats in the skim milk. The 18 carbon ketonic acid, licanic acid, was also tested and found to be effective. The odd chain fatty acid pentodecylic acid was not effective. The data support the generalization that even chain fatty acids containing 12 or more carbon atoms when fed with skim milk to the extent of 3 to 4 per cent are effective; those with less than 12 carbon atoms are not. It will be noticed that this interesting property of affecting the utilization of galactose is not related to the plant or animal origin of the fat but rather to a group of fatty acids that occur widely in natural fats.

*Relative values for growth.* This subject has recently had active investigation in several quarters and a controversy has developed regarding the interpretation of the several groups of data. Schantz, Elvehjem and Hart (43) performed experiments with groups of 21-day-old rats fed diets consisting of mineralized skimmed milk into which 4 per cent of fat had been added by homogenization. The respective diets contained as the variable fats of interest, butter fat, corn oil, cocoanut oil, cottonseed oil and soy bean oil. During the first few weeks of each experiment the rats receiving butter fat made better and more efficient gains in

body weight than did the animals eating the other fats, but this difference tended to disappear thereafter. In a later paper (44) experiments with fatty acid fractions of butter fat were reported. The respective fractions were the volatile, the unsaturated and the saturated acids. These were converted into their triglycerides and used as supplements to a diet of corn oil homogenized into the same mineralized and vitamin-supplemented skimmed milk. The animals receiving the acids representing the saturated-acid fraction exhibited a growth rate even greater than that observed when butter fat was the test fat. This claim was given further support by the data reported by Boutwell et al. (45) who stated that complete hydrogenation of the unsaturated acid fraction markedly increased its value for growth. These authors chose to regard their observations as favoring the view that there was present in the nonhydrogenated unsaturated acid fraction a long chain fatty acid (or acids) in unsaturated form which was rendered peculiarly effective for growth by hydrogenation; and that this material was apparently not present in either its saturated or unsaturated form in the various other oils tested because hydrogenation of these other oils did not improve their nutritive value when tested by incorporation into the mineralized and vitamin-supplemented skimmed milk. In this connection should be mentioned the claim by Boer (46) that butter fat contains a specific growth-prompting factor because his rats receiving butter fat grew better than those getting olive oil. On the other hand, Euler, Euler and Saberg (47) compared butter and margarine in diets otherwise adequate and observed more rapid growth in the rats subsisting on the margarine rations; these animals also had better hair coats and seemed to be more vigorous.

The next development of interest reported by the Wisconsin group of workers was that the nature of the carbohydrate in the ration plays a rôle. When the sole carbohydrate in the diet was lactose, animals receiving butter fat showed growth superior to that exhibited by the rats receiving corn oil (48, 49). When the carbohydrate was dextrose, sucrose, dextrin or starch, the growth of the corn oil groups was comparable to that of the butter fat groups or even better. The ration employed for these comparisons contained 32 per cent carbohydrate and 28 per cent fat. It was observed that, considered as a group, all of the lactose diets gave the poorer growth. This was explained by the assumption that lactose, as compared with the other carbohydrates, in some way adversely affects the intestinal flora, and that butter fat in some manner counteracts this effect either by supplying an essential growth factor—the unsaturated fatty acid or acids mentioned above—or indirectly by stimulating the bacterial flora to produce some unknown essential factor or factors. There is quite a literature available showing that the nature of the carbohydrate in the diet can affect the amounts of various members of the B complex found in the feces. Therefore the essential factor (or factors) being produced in accordance with this hypothesis might well be water-soluble vitamins instead of fat-soluble substances. These investigators also claimed that the younger the rat was when placed on the lactose-containing diet, the greater was the beneficial effect of the butter fat as compared with the vegetable oils.

In another paper these authors reported on comparisons of butter fat with many other fats and oils including several margarines (50). In these experiments the carbohydrate fraction was increased to 48 per cent because "unpublished work . . . showed that as the level of lactose was increased, the difference in growth rates between rats fed butter fat and those fed corn oil became greater." It pointed out that "this level of lactose is between the 32–40 per cent found in cow's milk and the 50–55 per cent found in human milk (dry basis)." For comparison with the lactose-containing ration there was used a diet containing a mixture of carbohydrates "modeled after the mixed carbohydrate diet such as man ordinarily consumes, and the results of this work led to an investigation of oleomargarines on similar rations." In all cases the animals on the mixed-carbohydrate rations, regardless of the fat used, exhibited better growth than those on the lactose diets. When lactose was the sole carbohydrate in the ration butter fat and lard were judged to be superior on the basis of the average weights of the six rats in each group at the end of 5 weeks on the diet. The authors did not give any data on the degree of variation characteristic of these averages, nor did they report any calculations made in an effort to confirm their impression that the differences cited as being significant really were significant. Some of the differences shown in their published graphs and tables seem, by mere inspection, to be large enough to be significant, whereas others are of questionable significance. In a matter of this sort the experienced critical judgment of the investigator deserves of course some weight. In view of the diametrically opposite results published by Deuel and others, however, these points of possible criticism seem worthy of note here.

Deuel and associates (51, 52) performed similar experiments with rats fed a diet of mineralized skimmed milk to which test fats were added and failed to observe any significant differences in the growth rates of groups of animals when seven different fats were used, namely, butter, margarine, corn oil, cottonseed oil, olive oil, peanut oil and soybean oil. In these experiments the diet contained 70.6 per cent mineralized skimmed milk, and 29.4 per cent test oil or fat. It will be noticed that the only carbohydrate in the ration was lactose. If the claim of the Wisconsin group of workers is correct, this should have resulted in the observation that butter fat is superior to the other tested fats, but such was not the case. The diet was supplemented with fat-soluble vitamins A, D and E; diacetyl, the substance shown by van Niel et al. (53) to be largely responsible for the characteristic aroma of butter and commercially available for use as a flavoring agent, was added to the various fats to secure equal intakes of the respective diets. These authors make the point that in the experiments by Schantz et al. (43) only carotene was used as a source of vitamin A with the vegetable oils, whereas the animals eating the butter fat diet must have received some vitamin A as such. To the extent that individuals are known to vary in their ability to utilize the provitamin carotene this criticism has merit. On the other hand, if the supply of carotene was very liberal, the animals doubtless secured sufficient vitamin A from this source and therefore this difference in the two sets of experiments can hardly account for the difference in the results.

Clausen, Barnes and Burr (54) have made interesting observations relating rate of growth to the rancidity of the diet that may have a bearing on this controversy. They were led to study the oxidation of the fat contained in experimental animal diets by the observation "that rats receiving a normal balanced diet containing the pure B vitamins in place of the customary yeast, cod liver oil, and lard, failed to grow and ultimately died. Replacement of the lard with other fats such as hydrogenated vegetable oils, corn oil, and butter fat provided an adequate diet for growth. If the pure B vitamins were replaced by yeast, normal growth resulted. On further investigation it was found that in the absence of the yeast, the lard and cod liver oil rapidly became oxidized and exerted a deleterious effect." These authors made the further interesting comment that "the inadvertent destruction of dietary essentials and the possible independent toxicity of rancid fat are factors that might confuse the interpretation of many dietary experiments." They definitely recommended that experimental diets be stored at low temperatures. In the experiments by Deuel et al. (52) great care was taken to exclude all rancid fat. The diets were prepared weekly and the unused supply kept in a refrigerator.

The question of the actual amounts of food eaten by the rats is important in studies of the type under discussion. Special efforts were made by Deuel and his collaborators to secure accurate food intake data. These authors criticized the Schantz, Elvehjem and Hart (43) study on the ground that the fat tended to separate out from the skimmed milk and therefore the composition of the food actually eaten at different times of the day was not the same. In their work reported in a later communication, however, Boutwell et al. (49) used dry artificial rations; such a criticism would not apply to this study. In feeding experiments involving butter one has to consider the extent to which the food intake is affected by the flavor of this food. Boutwell et al. (45) pointed out that the lower growth rate of the animals fed the corn oil diet as compared with those subsisting on the butter or butter fat fractions was associated with a smaller daily consumption of food. These authors chose to interpret this increased appetite and greater palatability as indicating a superior food. This was challenged by Deuel and associates (52) on the basis of the observation that rats "overwhelmingly prefer a diet of peanut oil to which commercial butter flavor has been added to the unflavored" peanut oil ration, and conclude that it is flavor rather than nutritive value that is determining the choice here. These observations on flavor were reported in detail in a later paper (55). Groups of 21-day old rats were given a chance to choose between two food mixtures over periods of 3 to 12 weeks. In the first series of tests the butter diet and one of the other rations containing the other fats (corn, cottonseed, olive, peanut or soy bean oils or margarine) were offered simultaneously. In every series of tests the butter diet was consumed in the larger amount. It was demonstrated that in the majority of instances rats prefer a complete diet the fat of which is butter to one where the fat is replaced by vegetable oils. When the choice was between fats flavored with diacetyl only and those containing commercial butter flavor of which diacetyl constituted 12 per cent, the latter was preferred. Tests with a labora-

tory prepared butter flavor containing monobutyrin, butyric acid and diacetyl to simulate commercial butter flavor gave similar results.

As a rebuttal to this argument that it was the preference of their weanling rats for the butter flavor, when fed ad libitum, with consequent greater intake of the butter diet that explained their finding that butter is a superior fat, Boutwell et al. (56) cited the results of experiments involving the removal of flavor from butter fat by chromatography or the addition of diacetyl to corn oil. Four diets were used, containing one of the following as the fat portion: (a) butter fat, (b) corn oil, (c) chromatographed butter fat, and (d) corn oil containing 0.0005 per cent diacetyl. When lactose was the sole carbohydrate, and the animals were fed ad libitum, the growth and appearance of the albino male rats was superior to that of the animals fed corn oil. Removal of the flavoring agents from butter, or the addition of diacetyl to corn oil, had little effect on the comparative nutritive value of the two fats. Comparisons of these fats using diets containing dextrose as the sole carbohydrate resulted in no difference, but in this experiment flavor was not the variable of interest. In their discussion these authors criticised the view that the paired-feeding technic has special merit and application in studies of this sort. They point out that paired-feeding "penalizes the superior food and ultimately obliterates the difference as measured by the growth rate and well-being of the experimental animal." This would doubtless be their comment on the study reported by Zialcita and Mitchell (57) who compared butter and corn oil with respect to growth-promoting value for baby rats paired according to litter, sex and body weight. The rats were forced-fed through a medical syringe adapted for the purpose. Because Boutwell et al. (48) had claimed that the younger the experimental animals the greater the differences obtained, Zialcita and Mitchell weaned one group at the age of one week and another at two weeks. Up to the age of three weeks they were fed an artificial liquid milk simulating in composition that of rat milk. One member of each pair received 15 per cent butter fat while the other received 15 per cent corn oil. After weaning age an artificial solid diet was used based on skimmed milk powder supplemented with casein, salt mixture and vitamins and containing 27 per cent of butter fat or corn oil. When the data were submitted to statistical analysis the differences were found not to be significant. The point made by the Wisconsin workers about paired-feeding operating to "obliterate the difference as measured by the growth rate" is readily illustrated by the data shown in the tables published by Zialcita and Mitchell. In the series of ad libitum feeding trials, the rats fed corn oil made better gains than those fed butter fat; however, the food consumption was likewise considerably greater. Zialcita and Mitchell concluded that "apart from differences in vitamin content, corn oil and butter fat are essentially equal in growth-promoting value for the rat." Deuel and Movitt (58) also performed some experiments with rats weaned when 14 days old. The growth of their animals was followed for a period of 12 weeks. The diets used consisted of the mineralized skimmed milk powder to which was added vitamin-fortified corn, cottonseed, peanut or soy bean oil, or whole butter or whole margarine. It was found that the efficiencies of conver-

sion of these diets to body tissue were similar. Moreover, the growth rates were similar to those of rats weaned at the usual age, namely 21 days. The prematurely weaned animals attained essentially the same bodyweights at later ages as normally weaned rats, thus showing that the premature weaning exerted no adverse influence.

The importance of considering the degree of variation characteristic of averages and subjecting data to mathematical analysis to determine significance of differences is further illustrated in the work of Freeman and Ivy (59) who observed during the first seven weeks of experiment better growth of weanling rats fed butter fat than of those receiving cocoanut oil, but this difference was not statistically significant; after three months, however, the butter fat fed rats were definitely larger. The observations of Harris and Mosher (60) can be regarded as confirming this "age effect". These investigators placed 120-day old rats on diets differing with respect to fat, one group getting cocoanut oil and the other group butter fat. The experimental diet was based on extracted milk powder, extracted yeast and the test fat which last-named item constituted 25 per cent of the basal mixture. Minerals and vitamins were also provided. The reader will notice that lactose constituted almost all of the carbohydrate in this ration; the amount of other carbohydrate furnished by the dried yeast must have been very small. The food was fed ad libitum. For the first 30 days the growth rates were practically the same. By the 60th day, however, the cocoanut oil-fed animals showed a slightly greater average gain. It is unfortunate that these authors gave no indication of the degree of variation characteristic of their published averages. It seems probable that the small difference noted was significant, but the critical reader would like to have more assurance on this important point.

Deuel and co-workers (52) offered as one of the possible explanations for the difference between their results and those reported by Schantz et al. (43) their own use of more animals in a group—at least 14—and their own great care in distributing members of a litter among the various groups to be fed the different test diets. In their work "there were three litters where the rats consistently gained far under the average on the various diets. When a small number of animals is used, variations might be noted especially if two from such a litter were assigned to one group." If some indication is given of the "spread" of the averages, any variation due to the use of such litters would receive some consideration in arriving at conclusions indicated by the data. It seems reasonable to assume that in the work of the Wisconsin group members of litters were properly distributed. On the other hand, the Wisconsin publications fail to indicate the "spread" of the averages and the differences are not subjected to statistical analysis, with the result that the independent critic is unable to determine for himself whether the various differences cited represent what might be expected as chance variations or are significant differences to be rightfully attributed to the presence or absence of lactose or the particular fat used.

If butter fat contains some unknown important factor as claimed by Schantz et al. (44) and Boutwell and associates (45), then this may have been present in all of the diets used by Deuel and co-workers, because these investigators used

unextracted skimmed milk powder. This could explain why Deuel et al. noticed no superiority of butter fat over the other fats and oils tested. The skimmed milk powder used by the Wisconsin workers was fat-free; their other rations which did not contain milk powder were purely artificial—so-called synthetic—combinations of food materials. This "explanation" has been challenged by Deuel (61) who points out that in the earliest tests reported by Schantz et al. (44) liquid skimmed milk was employed, and therefore no such extraction of lipids was employed, yet it was in these experiments that the most marked variations in growth were reported as occurring during a period of three weeks. Deuel calculates that the total daily intake of the hypothetical saturated fats of high molecular weight from the unextracted lipids of skimmed milk powder could scarcely exceed 10 mgm. "Since most of these fatty acids in this fraction are identical with those present in vegetable fats, those acids characteristic for butterfat and at present not identified would necessarily be present in extremely minute amounts."

Some observations pertinent to the theme under discussion have been made on calves. Gullickson, Fountaine and Fitch (62) fed groups of such animals, 2 to 6 in a group, for periods ranging from a few days to as long as six months. The following fats and oils were compared: butterfat, lard, tallow, cocoanut oil, peanut oil, corn oil, cottonseed oil and soybean oil. Each oil or fat was added to skimmed milk and the mixture then homogenized to yield a product containing 3.5 per cent fat. A control group received unhomogenized whole milk. Sufficient cod liver oil to supply vitamins A and D was given separately. In growth rate and appearance the whole milk group was definitely superior to the others, with the lard and tallow groups giving almost as good results; the groups receiving cocoanut oil and peanut oil came next in the rating; corn, cottonseed and soybean oils gave definitely unsatisfactory results, some of the calves in these groups not surviving the experiment and others being saved only by transferring them to the whole milk ration.

Another factor that may have a bearing on this controversy is the strain of animals used. It has been shown that strain differences exist among rats with respect to numerous biological characteristics. The "Yale" strain of rats has a relatively low tolerance for glucose (63, 64, 65); the adrenals of male rats of the "Yale" strain are significantly heavier than those of male "Wistar" rats (66); sodium chloride improves the low tolerance to glucose of the "Yale" strain (67).

There are strain differences in rats with respect to the time of onset and degree of incidence of cataract following the feeding of lactose (68). Still other illustrations might be cited but it is unnecessary to list them here. One that is of some interest in relation to the theme under discussion has been reported recently by Erschoff and Deuel (69). Male and female rats of the "Long-Evans" and the "U.S.C." strains were placed at weaning on purified diets containing lactose, beta-lactose, glucose, galactose, sucrose and corn starch as the sole source of carbohydrate. The animals failed to survive on the rations containing lactose or beta-lactose, those of the "U.S.C." strain living significantly longer, however, than those of the "Long-Evans" strain. These animals on the lactose

diets developed alopecia; their length of survival was correlated with severity and diarrhea; and a relationship was observed between degree of mortality and previous maternal diet. In discussing the significance of their findings Ersohff and Deuel cite literature dealing with the known effect of dietary lactose on the intestinal flora and the bacterial synthesis of vitamins of the B complex group, and the possibility that the change of the flora to the acidophilic type precipitates nutritional deficiencies either through failure of intestinal synthesis or utilization of factors already present. Boutwell et al. (48) and Geyer and associates (70) had suggested essentially the same possibilities. Examination of earlier diets in which lactose or beta-lactose served as the sole carbohydrate has revealed, according to Ershoff and Deuel,

"the fact that in almost every case natural sources of the vitamin B complex were employed in addition to unextracted casein and fats such as lard or Crisco. The possibility of nutritional factors being present in these materials in sufficient concentration to make good any deficiency resulting from alterations in the intestinal flora has not been excluded. The findings of Boutwell et al. (49, 56) and Geyer et al. (50) that butter fat and lard are superior to corn oil in promoting growth of rats receiving lactose as the sole carbohydrate would seem to indicate this very condition. It seems significant that Geyer et al. (50) reported their rats maintained on the 48 per cent lactose and 28 per cent corn oil ration as developing 'rough and discolored fur coats, blood-stained noses and scaly paws (when the humidity was abnormally high)'."

Similarly, Boutwell et al. (56) noted the occurrence of alopecia in rats eating a similar ration. The Wisconsin workers have stressed the point that more marked differences in growth due to fat are seen when high levels of lactose are fed, but it is at these high levels of lactose-feeding that the phenomena indicative of lack of some members of the B complex most readily occur; and, in view of the Ershoff and Deuel (69) observations, strains of rats can differ significantly in their ability to subsist on high-lactose rations without harm.

It seems evident that only further work on this problem by different groups of workers using the same rations, strains of animals, and as nearly the same experimental conditions as is possible can resolve this interesting controversy.

*Relative values for pregnancy and lactation.* The earlier literature on the relation of fat to lactation was reviewed in 1937 by Anderson and Williams (1). Much of it deals with the relation of dietary constituents to the production of milk in cows, goats and sheep, and the chemical composition of that milk. It has long been known that the food carbohydrate is a source of milk fat. Fat is also a suitable material, and within certain limits the percentage of fat in the milk is influenced by its content in the feed. Beger (71) showed that the feeding of butter fat to goats is associated with increases in the fat content of the milk. Allen (72) found that the feeding of whole milk to dairy cows in addition to the usual ration results in a marked increase in the yield of butter fat. The work of Williams and Maynard (73) on lactating goats emphasized that the feeding of a fat-free ration is associated with a rapid drop in milk yield and an equal or greater decline in fat yield; the effect of adding a fat supplement was always to arrest wholly or in part this decline, although butter oil and cocoanut oil increased the

fat content. The lack of fat per se thus appears to be an important factor. Anderson and Williams (1) make the statement that "much of the work on the feeding of palm kernel oil, cocoanut oil and butter fat to lactating animals lacks significance due to the fact that the augmented fat content of the milk resulted from lowered milk yield rather than to the influence of increased dietary fat." Maynard (74) has suggested a reason why the feeding of cocoanut oil meal and palm kernel oil meal high in fat can raise the fat content of the milk as reported by certain investigators. "These fats are rather unique as regards fatty acid distribution in containing, as does milk fat, a considerable amount of acids lower than $C_{16}$, though not containing the very low ones present in milk fat. Since butter itself is effective, perhaps a fat with a fatty acid distribution approaching it in some respects would have some effect."

In their series of papers dealing with the comparative nutritive values of fats Deuel and associates have one (75) reporting observations concerning fertility and lactation in animals that had been raised on various diets consisting of mineralized vitamin-fortified skimmed milk powder containing butter or on similar diets containing corn, cottonseed, olive, peanut or soy bean oil, or a margarine in place of the butter. No flavoring was added to the rations fed to the first series of animals. When the importance of flavoring became evident—discussed earlier in this review—diacetyl or commercial butter flavor was added to the rations used in the later series (II and III). The various diets were adequate for fertility shown by the fact that 94 per cent of the females cast litters. These authors comment: "If fat per se is required for fertility and pregnancy, its requirement may be equally well satisfied by the various vegetable fats as by butter. From the standpoint of lactation, as judged by the survival of the litter and the weight of the young at weaning time, the different fats were also equally effective." Olive oil represented a possible exception to this last statement, but because of the small number of rats receiving this fat, no conclusion is really justified with respect to it.

Maynard and Rasmussen (76) published observations on the influence of certain dietary fats on lactation performance in rats. Two experiments are reported. An application of the paired-feeding technic to the problem was devised. It is worth describing here. The mother rats had been fed a stock diet from weaning as well as through gestation. After the birth of the young, pairs of mothers were chosen which were equal in weight, and from whose litters six young for paired groups of nearly equal body weight could be selected. Whenever possible these paired groups were equalized as to sex. One mother was fed the high-fat diet, and the other, the low-fat diet, equalizing the calorie intake in accordance with the appetite of the one consuming the smaller amount. This proved to be the rat on the low-fat diet in almost all cases. The young were weighed daily for 17 days to provide growth records during the period when they were entirely dependent upon mothers' milk. This furnished a measure of lactation performance. The young were then sacrificed and analysed. In the first experiment 7 per cent of corn oil was the fat added to the low-fat ration to make the high-fat diet. In the second series a purified artificial low-fat diet was de-

vised; in order to make the high-fat diet, 15 parts of the corn starch were replaced by either crude cocoanut oil or crude corn oil. In both experiments the young from the mothers on the high-fat diet made better growth and contained more dry matter, fat and protein. The markedly different fatty acid make-up of corn oil and cocoanut oil had no detectable influence on the results. These findings agree with those reviewed earlier by Anderson and Williams (1) and already discussed. They have been extended by the work of Loosli et al. (77) who used the same technic and compared a fat-free diet with rations containing varying amounts of corn oil and hydrogenated cocoanut oil. The young suckled by mothers fed the corn oil diet made more rapid growth than those whose mothers ate the fat-free ration. Carcass analyses showed that the extra gain of the young consisted largely of fat. The diet containing hydrogenated cocoanut oil gave no better growth than the fat-free diet, and this could not be remedied by feeding ethyl linolate to the mothers or directly to the young. Similarly, feeding the mothers 125 mg of ethyl linolate each day when they were eating the fat-free diet did not improve the lactation response. The adverse effect of hydrogenating the cocoanut oil was therefore not due merely to saturation of any essential unsaturated fatty acids. This observation of poor performance with hydrogenated cocoanut oil does not agree with the results obtained by Deuel et al. (75) discussed previously. No obvious explanation is evident. It is obvious, however, that the technics employed were not quite the same.

Vinson and Cerecedo (78) have recorded some observations on growth, reproduction and lactation in rats maintained through four generations on highly purified diets. A diet containing 10 per cent fat in the form of corn oil was totally inadequate in supporting lactation; with hydrogenated cottonseed oil (Crisco) and lard present to the extent of 15 per cent of the ration, lactation was regarded as good and growth of the young considered to be normal. It was possible to get normal growth with the diet containing 20 per cent protein (casein) and 10 per cent lard, but this did not support lactation; when the protein was increased to 30 per cent, adequate lactation was obtained. During lactation on these purified diets the mothers lost considerable weight and this could be prevented by the feeding of yeast in contrast to the ineffectiveness of administration of biotin, para-aminobenzoic acid plus inositol, and yeast nucleic acid. The requirements of various specific factors for lactation still remain to be determined and the literature on this subject need not be reviewed here. It will be noticed that the levels of both fat and protein in the diets employed by Vinson and Cerecedo were shown to be important in relation to lactation performance. The experiments described were not devised to furnish a test of various fats with respect to nutritive value.

In the latest paper of their series Deuel and associates (79) report observations of successful growth and reproduction of rats over ten generations in which the lineage was through the first litter and of eight generations in which the lineage was through the second litter, where the diets were a modification of the Sherman diet B in which butterfat was replaced by vitamin A-fortified margarine fat. The Sherman B diet consists of two-thirds whole wheat and one-third whole milk powder. On such diets the growth rate considerably exceeded that ob-

tained with animals on a stock diet and progressively improved with the later generations. Sherman and Campbell (80) have reported the ability of their B diet to support excellent growth of the young, maintenance in health of the adults, and the requirements of reproduction including lactation over many generations, a decrease in the infant mortality observed with the A diet (consisting of one-sixth whole milk powder and five-sixths whole wheat), and an increase of 10 per cent in the average length of life of the adults compared with that observed with the A diet. Obviously the B diet is an excellent one. Deuel et al. (79) concluded "that a vegetable fat such as that contained in a margarine can serve adequately in place of butter fat for growth and reproduction on a diet otherwise nutritionally satisfactory." These authors considered that their "results answer in the affirmative the question raised by Boutwell et al. (48) as to the adequacy of a vegetable fat for continued growth and reproduction over a number of generations." To this reviewer it seems pertinent to point out that, as shown in the paper, these results were obtained with a diet containing extracted skimmed milk powder and the diet was supplemented once weekly with 5 grams each of lean beef and lettuce. The conclusion reached by these authors, as stated above, is doubtless justified especially when the qualifying words "on a diet otherwise nutritionally adequate" are used and when one considers the varied dietary conditions pertaining to man that commonly prevail. Experiments of this sort involving the use of the Sherman B diet or modifications of it, however, can hardly be regarded as critical in nature in resolving the controversy raised by the claim that butter fat has a special value in nutrition when lactose is the sole carbohydrate in the diet. Their significance for practical nutrition is quite evident however.

## SUMMARY

Edible fats the melting points of which are not too high to prevent liquefaction in the alimentary tract are digested and absorbed to about the same degree. Such differences as have been found are of no practical nutritional significance.

Edible fats differ with respect to their value as a source of known vitamins. Fish oils contain vitamin A as such; the vitamin A potency of butter is due partly to vitamin A and partly to the provitamin carotene; the vitamin A potency of plant oils is an expression of their content of carotene and related biologically effective carotenoids.

With respect to vitamin D, butter contains variable amounts; the margarines are practically devoid of it unless fish oils containing this factor are used to supply vitamin A for fortification; natural foods which contain vitamin D are of animal origin.

The oils of plant embryos are the richest natural sources of vitamin E; the amount of this vitamin in margarines depends upon the formula used; butter contains small but significant amounts.

Little can be said regarding the amounts of vitamin K present in plant and animal fats; any slight differences are probably of no nutritional significance. It has been claimed that cream contains an unknown factor essential for the guinea pig.

Natural fats differ with respect to their content of the essential unsaturated fatty acids but the amounts needed by the organism are so small that these differences are probably of no practical nutritional significance.

Natural fats have not been found to differ appreciably in their effects on the body's needs for other dietary essentials.

With respect to the value of various fats for growth: evidence has been offered by one group of investigators that when lactose is the sole carbohydrate in the food, butter fat is slightly superior to margarines and various oils of plant origin, but this view has been vigorously challenged by other workers; with diets similar to the so-called mixed diets used by man such fats have essentially the same nutritive value. The lack of fat per se results in lowered milk production by the lactating animal. There is some evidence that lactation performance is not as good when the organism is fed hydrogenated cocoanut oil as compared with that when the natural cocoanut oil is fed; this difference is not obviated by supplying essential fatty acid. In a diet otherwise nutritionally satisfactory, a vegetable fat such as that contained in a margarine can serve adequately in place of butter fat for growth and reproduction, shown by experiments with eight and more successive generations of rats.

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# INTERFERENCE WITH BIOLOGICAL PROCESSES THROUGH THE USE OF ANALOGS OF ESSENTIAL METABOLITES

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Until quite recently the study of the relation of chemical constitution to biological action proceeded along pathways well established by precedent. Observations made during routine testing of compounds, or often by chance, served as the basis for the synthesis of various types of chemically related substances. In this manner many substances of great practical utility were found, and in the same manner many more will undoubtedly be found in the future. Also, through this type of approach, much information has been accumulated which is significant to the correlation of pharmacological action with various chemical and physical characteristics in series of compounds. Only rarely, however, has information so acquired been sufficient, or has our knowledge of the mechanism of action proved adequate, to permit the purposeful design of new compounds of greater effectiveness. These empirical methods of approach, although by no means unfruitful, are tedious and only slowly productive of results; it is encouraging to find, therefore, among recent studies, evidence that elements of logic may now be admixed with empiricism.

The development of these newer concepts stemmed directly from the discovery of the sulfonamides and the partial explanation of their mode of action. Accordingly, it is desirable to review the more important findings upon which our present concepts of the action of sulfonamides are based.

Early in the study of the action of sulfanilamide it became evident that its effectiveness is not dependent upon a stimulation of the defense mechanisms of the body or on a simple germicidal action in the ordinary sense. Thus, the presence of a low concentration of sulfanilamide may have little effect for several hours on the rate of reproduction of microorganisms *in vitro*, although ultimately a bacteriostatic action is evidenced.

The effectiveness of sulfanilamide was shown to be dependent, within certain limits, upon the concentration of the drug, but other factors of importance have been demonstrated. Among these are the phase and strain of the bacterial species, the size of the inoculum, and particularly the composition of the culture medium. These facts led McIntosh and Whitby (1) in 1939 to suggest that the delay in onset of sulfonamide action might be due to "a slow combination or neutralization by the drug operating on some essential food substance or enzyme." Even earlier (1938), Lockwood (2) concluded from experiments with peptones that "the population curve in any medium is a resultant of the combined activities of peptone and sulfanilamide," and suggested that the drug might interfere

[1] This review is based on an earlier paper prepared while the author was a member of the staff of the Medical-Research Division of Sharp & Dohme, Inc., Glenolden, Pennsylvania, and presented before the New Jersey section of the American Chemical Society and the A.A.A.S. Gibson Island Conferences of July, 1944.

with the production or activity of certain enzymes. It was his thesis that the enzymic degradation of proteins accomplished by certain invasive organisms is prevented by sulfanilamide, but that the various lytic products, such as peptone, by supplying nutritive material, could abolish the effectiveness of the drug.

A major portion of the inhibitory action exerted by various peptones, and by extracts of yeasts, bacteria, and various tissues, can now be accounted for largely by their content of a specific substance, the action of which is antagonistic to the effect of sulfanilamide, and its various active derivatives, and upon which the life processes of many microorganisms appear normally to be dependent. Prominent among those workers whose findings justify this conclusion are the Englishmen, Stamp, Woods, Fildes, and McIlwain, and the Americans, Blanchard, MacLeod and Wood.

Stamp (3) showed in 1939 that heat-killed bacterial cells, as well as extracts of them, enabled homologous "organisms to grow in dilutions of sulfanilamide normally producing complete inhibition." He found that the activity of such extracts was not destroyed by heat, acids or alkalis and suggested that split products of protein might be responsible, although the possibility that the factor supplied might be "a substance of the nature of a coenzyme . . . ." was also given emphasis. Stamp's studies, however, did not appear adequately to exclude the possibility that his anti-sulfanilamide factor functioned simply as a stimulant of bacterial growth, rather than as a specific inhibitor of the action of sulfanilamide.

In 1940 Green (4) reported that an extract of *Brucella abortus* organisms greatly accelerated the rate of reproduction of homologous organisms in the presence of sulfanilamide. Addition of such bacterial extracts to cultures which appeared to have been sterilized by the previous addition of sulfanilamide caused a resumption of growth, while the bacteriostatic action of thionin was not antagonized by the extracts. However, Green was unable to demonstrate the presence of the anti-sulfanilamide factor in peptone or in extracts of other materials.

The studies of MacLeod (5), on the other hand, showed that a variety of peptones and tissue-extracts are capable of exerting marked anti-sulfonamide action. In certain tissues he found only small amounts of sulfonamide-inhibitor when the material was fresh, whereas after autolysis a relatively large amount was present; in human urine only a trace of activity was found prior to hydrolysis with acid. A possible clue to a mechanism of resistance to sulfonamides was uncovered by MacLeod, who noted that the bacterial production of sulfonamide-inhibitor was markedly increased, as resistance to sulfapyridine was developed in a strain of virulent pneumococci (type I).

In 1940 Woods and Fildes announced, in a preliminary report (6) which was expanded later by Woods (7), that *p-aminobenzoic acid* is enormously active as an anti-sulfonamide factor. A concentrate prepared from yeast was found to contain a factor resistant to destruction by heat, acid or alkali. The active substance was destroyed by nitrous acid and was diazotizable; its effectiveness, which was lost on acetylation or esterification, was regained following hydrolysis of such derivatives. These properties, together with measurements of solu-

bility and acid-dissociation, led logically to a trial of synthetic p-aminobenzoic acid. Although isolation of the naturally occurring inhibitor was not accomplished by Woods and Fildes, this was soon reported by Rubbo and Gillespie (8), who obtained from 30 kgm. of wet yeast 2 mgm. of a five-times recrystallized, benzoylated material with the proper melting point for p-benzoylaminobenzoic acid; an identification which was not absolute. Blanchard (9), suspecting that much of the inhibitor in yeast is in a combined form, allowed fresh baker's yeast to autolyze with ethyl acetate prior to extraction, obtained the factor in pure form and identified it conclusively as p-aminobenzoic acid. He suggested that in nature the substance exists largely in a combined form in which the p-amino group is blocked, perhaps in a manner resembling a peptide linkage.

In a paper accompanying that of Woods (7), Selbie (10) showed that the anti-sulfonamide effect of p-aminobenzoic acid (*paba*) is not restricted to the test tube, since the chemotherapeutic action of sulfanilamide, in mice infected with a lethal strain of hemolytic streptococci, is also nullified by the administration of paba. The significance of the factor to the metabolism of certain microorganisms was first demonstrated by Rubbo and Gillespie (11), who found that with these organisms reproduction fails unless paba is present in their environment. However, as is evident from a number of investigations, the majority of the bacterial species susceptible to the action of sulfanilamide and its derivatives is not dependent upon external supplies of paba, since such organisms are able to synthesize adequate amounts of the material.

In 1940 Fildes (12), in whose department much of the work which led to our present concepts was done, discussed the significance of the data available at the time and suggested that paba should be looked upon as an "essential metabolite," with the utilization of which sulfanilamide and its derivatives interfere. Although for several reasons this term is not altogether satisfactory it has come into fairly general use. *Essential metabolites* may be defined as obligatory components of the normal sequence of metabolic reactions occurring in a living cell; they consist of two main types: *endogenous*, which are supplied by the synthetic activities of the organism, and *exogenous*, which an organism must obtain from its environment, although they are produced by the metabolic activity of other forms of life. Essential metabolites of the latter type are often referred to as "*essential growth factors*," and, of course, for those species requiring them, include those substances commonly referred to as vitamins. Under certain conditions essential metabolites may be synthesized slowly, but at a rate incompatible with optimal performance. Such a circumstance may enable an organism to survive under poor environmental conditions, but to thrive luxuriantly when the need for synthesis is obviated.

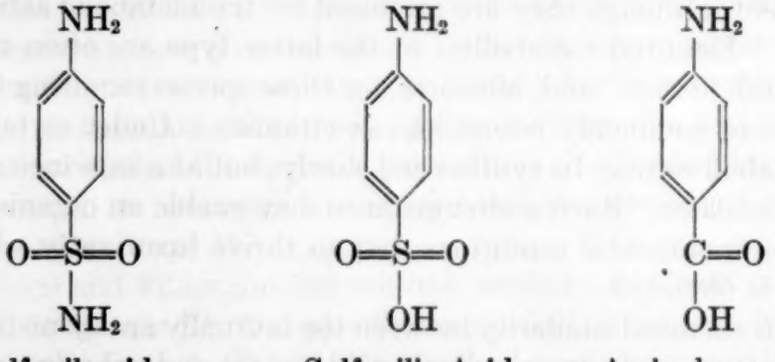
The obvious chemical similarity between the mutually antagonistic substances, sulfanilamide (p-aminobenzene-sulfonic acid amide) and paba (p-aminobenzene-carboxylic acid), suggested immediately that those components of the bacterial cell which normally combine with paba may also have an affinity for sulfanilamide. In admixture the two compounds would thus enter into competition for such components, and as a result two products would be formed, one presumably

essential to the course of a vital process, and the other largely or entirely inactive in that respect. Dependent on the ratio of the analog (sulfonamide) to the essential metabolite (paba), the physiological response would vary from growth and reproduction of a normal character to the complete obstruction of a reaction essential to life or reproduction. This view of the interference with the function of an essential metabolite, through the formation of a compound composed in part of a metabolite-like substance, is quite analogous to the combination between an enzyme and its substrate or a substrate-analog, that is, a compound in some respect chemically similar to the substrate. With many enzymic systems, the normal and the abnormal compound antagonize one another competitively and the kinetics of the reaction usually can be explained with mathematical precision.

The term *competitive inhibitor* can be applied to the mutually antagonistic components of a system in which the rôle of either the metabolite or the anti-metabolite is reversibly and competitively interfered with by its antagonist, the relative amounts of the two substances in combination with the enzymic or other cellular components being dependent upon their relative affinities for them and upon their concentration-ratio.

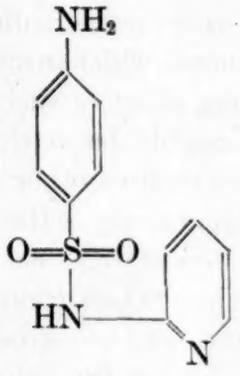
A simple analogy may be drawn if we picture the natural substrate or metabolite as a key, so notched and grooved as to fit perfectly the mechanism of a lock which it operates smoothly and efficiently. The interfering substance, anti-metabolite, metabolite-analog, or enzyme-antagonist, may then be characterized as an imperfect key which, by virtue of its similar grooves, is able to enter the lock but is unable to operate its mechanism, although by its position in the lock it interferes with the entrance of the proper key.

According to this view the more closely a sulfonamide resembles paba in those properties which enable it to react with some specific constituent of bacterial cells, without forming a functional complex, the more efficiently may it be expected to function as an anti-metabolite or blocking agent. At first glance it might appear that these requirements should be satisfied better by sulfanilic acid than by sulfanilamide, and certainly more closely by these compounds than

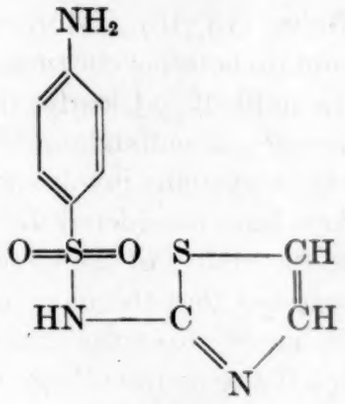



Sulfanilamide Sulfanilic Acid p-Aminobenzoic Acid

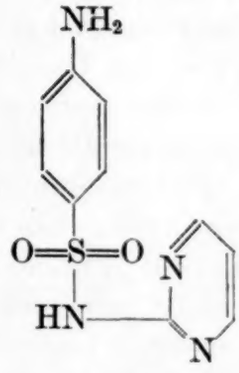
by the more active, but more complicated, heterocyclic derivatives of sulfanilamide, such as sulfapyridine, sulfathiazole, sulfadiazine and sulfamerazine. It has now been demonstrated, however, that those substances derived from sulfanilamide which are more effective chemotherapeutically than is sulfanilamide are also much more efficient in interfering with the action of paba, and no com-



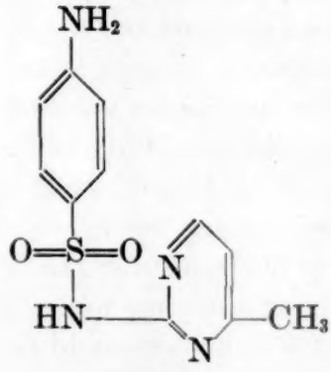
Sulfapyridine



Sulfathiazole



Sulfadiazine



Sulfamerazine

pound has been found, which appears to behave like sulfanilamide, that is not effectively inhibited by paba.[2]

Extensive study of the physico-chemical properties of compounds antagonistic to paba has led, during the last two or three years, to marked advances in our concepts of those structural characteristics which enable compounds to compete effectively with paba for the components of bacteria which utilize that substance. At one time it was considered that the greater activity of some derivatives of sulfanilamide containing heterocyclic ring-substituents in the $N^1$-position, might be due to the ability of such derivatives to inhibit other systems, in addition to those involving paba. The possibility of a "double-barreled" action will not be thought unreasonable, when it is considered that nicotinic acid, an important essential metabolite, contains within its molecule the pyridine ring, found also in sulfapyridine. Further, in thiamine there are found the thiazole and the pyrimidine rings. These heterocyclic rings find their counterparts in sulfathiazole, on the one hand, and in the pyrimidine derivatives, sulfadiazine, sulfamerazine and sulfamethazine, on the other. Despite the interesting features of this theory, it now appears well established that although certain $N^1$-heterocyclic groups may indeed confer demonstrable accessory inhibitory characteristics upon sulfanilamide (West and Coburn, 13; Dorfman and Koser, 14), these do not contribute significantly to the chemotherapeutic activity of the compounds *in the concentra-*

[2] The properties of 'Marfanil,' $H_2N \cdot CH_2 \cdot C_6H_4 \cdot SO_2 \cdot NH_2$, differ sufficiently from those of sulfanilamide as to suggest that different mechanisms are involved in the bacteriostatic actions of the two compounds.

*tions found effective* (15, 16). Moreover, several very active sulfonamides are known which contain heterocyclic rings, the occurrence of which in microorganisms is believed to be unlikely. Clearly, the potentiating effect of such $N^1$-substitutions on the activity of sulfanilamide cannot reasonably be attributed to contributory effects on systems involving essential metabolites other than paba.

Several workers have considered the bacteriostatic activity of the sulfonamides to be related to the ability of these compounds to dissociate as acids. Bell and Roblin (17) suggested that the more negative is the —$SO_2$— group of a sulfonamide, the more closely does the group resemble the —$CO_2$— group of paba at pH 7, at which pH the metabolite is over 99.9 per cent in the anionic form and the —$CO_2$— group possesses a strongly negative charge. These workers showed that in a buffered medium of pH 7, *in vitro*, the bacteriostatic activity of a group of sulfonamides increased as the pKa increased, until maximal activity was attained at pKa values ranging between 6 and 8.5. Within their series of compounds bacteriostatic activity fell off steadily with further increase in the values for pKa. Since the negativity of the —$SO_2$— group is greatly increased by the loss of a proton from the adjacent nitrogen atom, the ionic form of any sulfonamide, according to this theory, would be much more active than the molecular form, and hence bacteriostatic power, as measured at pH 7, would increase with acid strength until complete ionization was attained. Compounds having pKa values of about 6 or higher would be essentially completely ionized at pH levels of 7 or above. The decrease in bacteriostatic activity observed with further increases in the values for pKa was considered by Bell and Roblin to be related to a second influence, namely, that acid strength is proportional to the electron-attracting power of the $N^1$-substituent. As this power increases beyond values sufficient to permit complete ionization at pH 7, the substituent group would attract electrons away from the —$SO_2$— group, thus decreasing its negativity, and hence the bacteriostatic activity of the compound.

Although the theory of Bell and Roblin, as tested by their experimental procedures, appears to have certain limitations, in that it does not appear entirely satisfactorily to account for the observed activity of non-acidic compounds, their concepts unquestionably mark a real milestone in progress along the road of correlation between chemical constitution, physical properties, and pharmacological action.

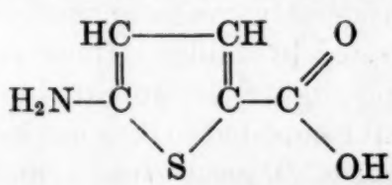
A further extension of this concept of the relation of structure to activity in the group of compounds which antagonize paba is to be found in the recent papers of Kumler and his associates (18, 19, 20). Although their concepts cannot be dealt with in detail, these workers place emphasis on the relation of resonance to the negative character of the —$SO_2$— group. The theory is said to account satisfactorily for the activity exerted by certain compounds that are incapable of ionizing as acids, and which form exceptions to the theory of Bell and Roblin. Kumler and his associates also suggest that the neutral molecules of ionizable sulfonamides may more efficiently reach the site of action, where "the ion then interferes with essential metabolic processes resulting in bacteriostasis." The conclusions reached by Kumler and his associates cannot be taken as final, since they have been challenged and disputed in recent articles by Bell, Bone and

Roblin (21) and by Bordwell and Klotz (22). Mention should also be made of the paper by Klotz (23) in which activity is related to acidity and to the effect of mass action upon an enzyme system.

It is known that in the blocking of the utilization of paba by one organism, *in vitro*, one derivative of sulfanilamide may prove definitely superior to another, while with some other bacterial species the relative merits of the two compounds may be quite reversed. This does not signify necessarily that our basic concepts of competitive inhibition are incorrect, but rather that our understanding of the various complicated processes involved, such as penetration, adsorption and absorption, is far from complete. For example, it is not unreasonable to consider that the relative insensitiveness of the tubercle bacillus to the various ionizable sulfonamides may be attributable to the effect of the lipoidal constituents of this microorganism on the penetration of the drug.

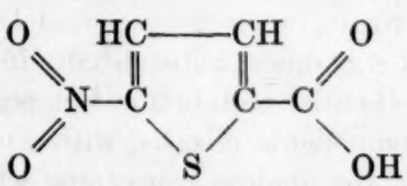
The important fact has been mentioned previously that several compounds related chemically to paba which have non-acidic properties quite different from those of sulfanilamide, e.g., certain sulfones, aminobenzils, aminoacetophenone, N-methyl compounds, and the like, possess bacteriostatic properties which are blocked by paba. Additional evidence for the concept of bacteriostasis through the antagonism of paba has been advanced by the studies of Johnson, Green and Pauli (24), who found that ring-substituted, and other derivatives of p-aminobenzoic acid are anti-bacterial *in vitro*, and that their bacteriostatic activity is blocked by the addition of paba; none, however, is as active as sulfanilamide. Particularly interesting were their studies of various new compounds in which the thiophene ring was substituted for the benzene ring of paba. These analogs are comparable to a modification of thiamine (to be discussed later) in which its thiazole ring is replaced with the pyridine ring, yielding a compound (pyrithiamine) which effectively antagonizes the utilization of thiamine. In one case a —CH=CH— is replaced with —S—, in the other the change is in the reverse direction.

The thiophene analog of paba, 2-amino-5-carboxythiophene (aminothiophenic acid), is too unstable to test directly, and only derivatives or related compounds

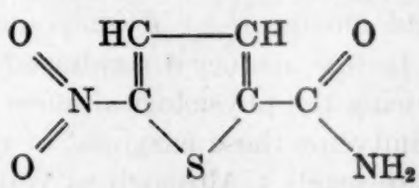


2-amino-5-carboxythiophene

have been studied. Johnson et al. (24) found, however, that the bacteriostatic action of the corresponding nitro compound and its amide is blocked by paba and

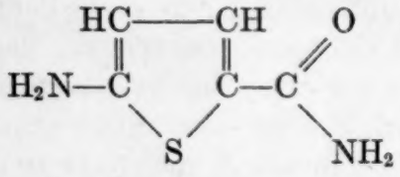


2-nitro-5-carboxythiophene



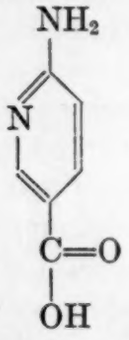
Amide of 2-nitro-5-carboxythiophene

compares favorably with the activity of the most active sulfonamides, at least on *Streptococcus hemolyticus, in vitro*. Since the reduction product derived from the bacteriostatically active amide of 2-nitro-5-carboxythiophene, namely, the amide of 2-amino-5-carboxythiophene, is inexplicably inactive, Johnson and his co-



Amide of 2-amino-5-carboxythiophene

workers suggest that some reduction product of the nitro group, which more closely resembles an aromatic amino group, may be responsible for the bacteriostatic activity of the nitro compound. Although replacement of the benzene ring of paba with the pyridine ring yielded a compound with considerable activity



2-amino-5-carboxypyridine

against *E. coli* and streptococci, it was, for reasons at present unknown, unable to influence the metabolism of pneumococci. Analogs derived from thiazole or from furane were apparently too remotely related to paba to exert activity.

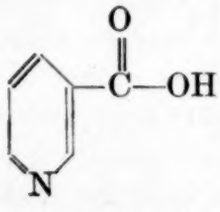
Miller (25) has also tested certain thiophene derivatives related to paba, with results in essential agreement with those of Johnson et al. (24). Since Auhagen (26), using a species of *Lactobacillus*, reported that p-aminobenzoylglutamic acid possesses considerably greater antisulfonamide activity than paba, this compound was also tested by Miller. However, with *E. coli*, under the conditions of the experiments, its activity appeared to be less than that of paba. Williams (27) also found this compound to be much less active than paba, when tested on *E. coli*, *S. hemolyticus*, *D. pneumoniae I*, and *Cl. acetobutylicum*.

The possibility that Woods' discovery of the antagonistic effect of paba on the action of sulfanilamide might have general significance was immediately apparent to several workers. In 1940 Fildes (12) suggested the possibility that new chemotherapeutic agents might be developed through the preparation of various structural modifications of compounds known to function as essential metabolites. In this manner it was hoped to find substances substantially incapable of producing the physiological effect of an essential metabolite, but possessing high affinity for those enzymes, or other components of cells, with which the metabolite reacts. Although as yet none of the analogs concerning which re-

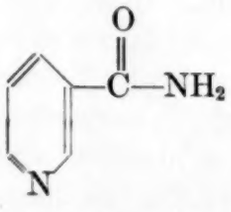
ports are available has given rise to a new series of practical chemotherapeutic agents, there is as yet no apparent reason why, in the more or less blind groping which led to the discovery of "Prontosil," science should have stumbled upon the only metabolite of microorganisms the function of which can be effectively antagonized within the body of an infected host. In this connection there is only a very remote possibility that antibiotic agents, such as penicillin, function as antagonists of essential metabolites; probably, however, because of the minute quantities which are required, there occurs some more subtle interference with enzymic systems vital to the continued function of a variety of microorganisms.

Quite logically the first attack on the problem of expanding the concept of metabolite-antagonism, was directed toward structurally identified metabolites which, although essential to the metabolism of microorganisms, were known to be required in very small amounts. Particularly active and effective in such studies have been McIlwain in England, and Woolley and Snell in this country.

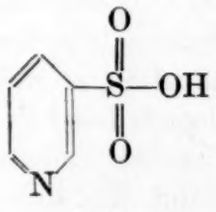
*Nicotinic acid analogs.* In 1940 McIlwain (28) described studies with pyridine-3-sulfonic acid and its amide, showing that these compounds effectively inhibit various bacterial species for which nicotinic acid, nicotinamide, or certain complexes of them, are essential growth factors.



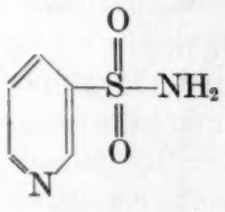

Nicotinic Acid




Nicotinamide




Pyridine-3-sulfonic acid

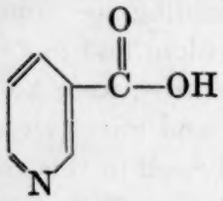



Pyridine-3-sulfonamide

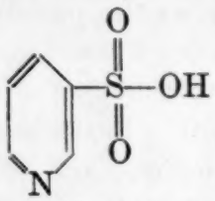
In contrast to the antagonism between paba and the sulfonamides, it appears that these nicotinic analogs are unable to interfere with the metabolism of those bacterial species which obtain their supply of derivatives of nicotinic acid by synthesis. Conceivably this inactivity may reflect a failure of the analogs to permeate the bacterial cells and to attain a sufficient concentration in that portion of the cytoplasm where nicotinic compounds or their complexes are synthesized. According to this view, effective inhibition by the analogs of those bacteria requiring an external source of nicotinic acid, or its amide, would appear to be dependent on an interference with the absorption of the growth factor by the organism. Whatever the mechanism, it is clear that other factors operate in addition to that of a simple competition between the metabolite and its antagonist, based on the relative numbers of the molecules of each and their relative affinity for the cellular component with which they react. McIlwain found from his studies of the effect of pyridine-sulfonic acid and its amide on the growth of staphylococci, and on species of *Proteus*, that there is a marked lack of equivalence of nicotinic acid, nicotinamide, and its derivative, diphosphopyridine nucleotide. In some cases, for example, inhibition with pyridine-3-sulfonic acid was more intense than with its amide, and it was tentatively suggested that the

sulfonic acid may affect most powerfully a reaction at some unknown stage higher than cozymase.

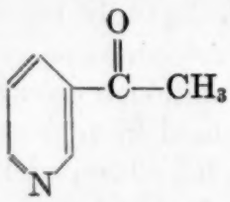
Since several animal, as well as bacterial, species require an exogenous supply of nicotinic acid or its amide, it would be of interest to consider whether the administration of pyridine-3-sulfonic acid to animals interferes with the utilization of the vitamin. Woolley et al. (29) in 1938 examined a series of substances for nicotinic acid-like activity in dogs with experimentally induced black tongue, and found that although pyridine-3-sulfonic acid and β-acetyl-pyridine produced none of the actions of nicotinic acid as a vitamin, they did exert a markedly toxic



Nicotinic acid



Pyridine-3-sulfonic acid



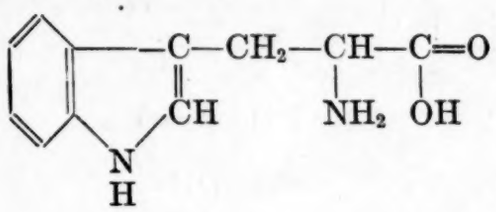
β-acetylpyridine

or even lethal effect in the deficient animals. Such effects, significantly enough, were not seen when either of the compounds was given, in similar doses, to dogs ingesting adequate amounts of nicotinic acid. In the light of present knowledge, these results strongly suggest that the two synthetic derivatives of pyridine inhibited the proper utilization of nicotinic acid by dogs whose stores of the vitamin were severely depleted and that rapidly toxic effects were thus produced. In healthy animals, however, the supply of nicotinic acid derivatives was adequate to compete with and thus to prevent the blocking effects of the analogs. It is possible that if sufficiently large amounts of the compounds were given, interference with the nicotinic acid metabolism of the normal dog could be accomplished. A reinvestigation of this subject might prove profitable.

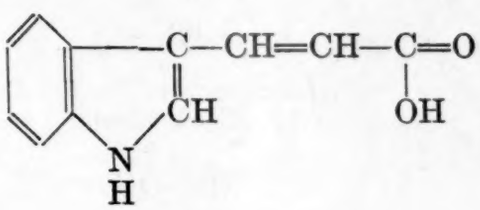
Woolley and White (30) showed that in the mouse, which appears to fulfill its requirements for nicotinic acid derivatives by synthesis, pyridine-3-sulfonic acid, in the dosage used, produces no evidence of deleterious effects. These workers pointed out that this failure may be due to a mechanism similar to that responsible for the lack of bacteriostatic effect on microorganisms which synthesize their own supply of nicotinic derivatives. A very recent report (31) indicates, however, that nicotinic acid deficiency can be produced in the mouse with β-acetylpyridine, so that the explanation for the failure with pyridine-3-sulfonic acid is not readily apparent. Possibly, however, differences in the penetration of cells by β-acetylpyridine and pyridine-3-sulfonic acid are responsible for the difference in their activity; it will be noted that the former is non-acidic while the latter is an ionizable acid.

*Amino acid analogs and pyridoxine.* In 1941 McIlwain (32) found that certain aliphatic α-aminosulfonic acids inhibit a number of microorganisms by virtue of their structural similarity to various α-aminocarboxylic acids. It is worth noting that this type of antagonism is unspecific, since the effect of an α-aminosulfonic acid is removed by α-aminocarboxylic acids other than that which is strictly analogous to the inhibitor. McIlwain pointed out that the "Production

of a compound capable of interfering with a particular metabolite evidently demands consideration of properties of which structural formulae give only approximate indications," a statement the truth of which is becoming increasingly evident, particularly, of course, from the study of the sulfonamides, as has been discussed previously.



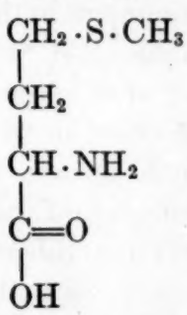
Tryptophane



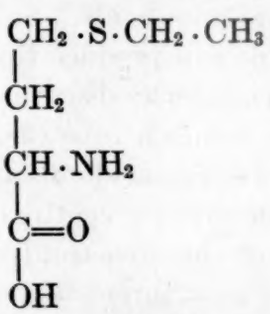
Indoleacrylic acid

Fildes (33) showed in 1941 that a compound resembling tryptophane, indoleacrylic acid, in a concentration of M/8000 to M/1000, will block the growth of *E. coli* on an ammonium lactate medium; this effect is reversed by tryptophane, but there is no quantitative relationship between the effect of the two compounds. From this fact and because the addition of only a trace of tryptophane causes growth in spite of the inhibitor, Fildes suggested that indoleacrylic acid interferes with the synthesis of tryptophane, rather than with its utilization.

It is interesting to note that a phenomenon probably to be attributed to metabolite-antagonism was recorded in 1938 by Dyer (34), in duVigneaud's laboratory. In feeding experiments, ethionine, the S-ethyl homolog of methionine, was unable to support the growth of rats on a diet deficient in cystine and



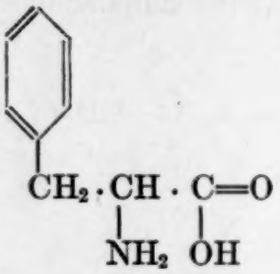
Methionine



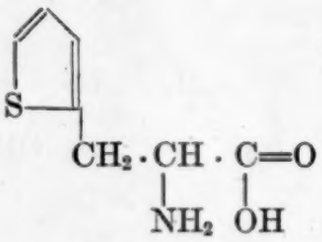
Ethionine

methionine, and also caused certain toxic manifestations. Animals given the homolog showed a loss in appetite, lost weight more rapidly than rats on the basal diet alone, and died after short periods of supplemental feeding. When methionine and ethionine were administered together, the loss of appetite was less marked and the animals usually gained weight, although not as rapidly as when ethionine was excluded from the diet. It now appears reasonable to explain these results on the basis of competitive inhibition of the utilization of methionine by its higher homolog. Conceivably the compound interferes with the ability of methionine to serve as a donor of methyl groups although other functions of the amino acid may be affected as well. Harris and Kohn (35) have observed that ethionine limits bacterial growth, apparently by interfering with the bacterial metabolism of methionine.

The metabolism of phenylalanine, an amino acid essential for growth in the rat, has been shown by duVigneaud (36) to be affected deleteriously by thienylalanine, a compound in which the benzene ring of phenylalanine is replaced by



Phenylalanine

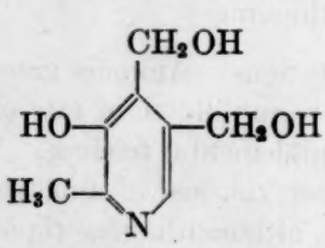


Thienylalanine

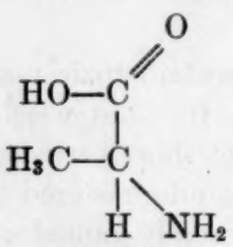
the thiophene ring. It was found that the growth of rats is inhibited by the inclusion of this isostere in the diet, an effect competitively antagonized by the naturally occurring amino acid. The growth of yeast also served to demonstrate the competitive antagonism between phenylalanine and the isostere.

There are several examples of metabolite-antagonism involving amino acids and other growth factors which have not yet been completely explored. Snell and Guirard (37) showed that under certain circumstances the requirement of *Streptococcus lactis R* for pyridoxine can be completely satisfied by α-alanine. Excessive amounts of glycine, however, and to a lesser extent of serine or of threonine, inhibit the growth of this organism; these inhibitions can be removed, within limits, by pyridoxine, and even more effectively by alanine, but not by other vitamins or amino acids. Snell and Guirard comment on the fact that both glycine and serine can produce toxic effects in animals. At Snell's suggestion, Fishman and Artom, who discovered the toxic effect of dl-serine when administered to rats by stomach tube (38, 39), studied the effect of pyridoxine on the toxic effect of the amino acid (40) and found that, of the various vitamins tested, it is the most effective preventive of the toxic manifestations caused by serine.

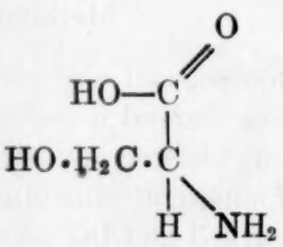
Examination of the structural relationship between pyridoxine and alanine or serine shows a most interesting similarity, as has been pointed out by Snell.



Pyridoxine



α-Alanine



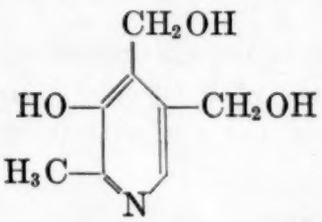
Serine

Despite the structural similarities the mechanism responsible for the antagonism between pyridoxine and alanine or serine is not known. Speculation is probably unwise at present, in view of the complications introduced by Snell's brilliant elucidation of the chemical nature of pseudopyridoxine (41), which has now been resolved into two compounds, pyridoxamine and pyridoxal, both closely related

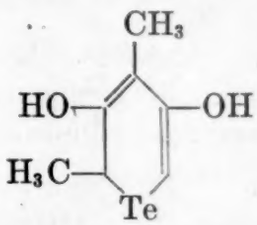
to pyrodixine and both now available in synthetic form as a result of the work of Harris, Heyl and Folkers (42).

Study in animals of various antagonists of the essential amino acids, and of other compounds important for structural as well as purely functional reasons, should prove to be most helpful in uncovering much that is now hidden. As possible chemotherapeutic agents, however, such compounds offer less promise, since the relatively large requirement of the animal body for these nutrients greatly reduces the possibility of their analogs having practical utility.

Another phenomenon possibly involving the action of pyridoxine on bacteria was postulated by Gulland and Farrar (43), who found that certain very toxic compounds resembling pyridoxine in structure, but containing tellurium in place of the nitrogen of the pyridine ring, were effective bactericidal agents, *in vitro*, against *E. coli*, *B. typhosus*, *Staph. aureus*, and *Strep. hemolyticus*. The structural formulae of pyridoxine and of their most active compound of tellurium are:

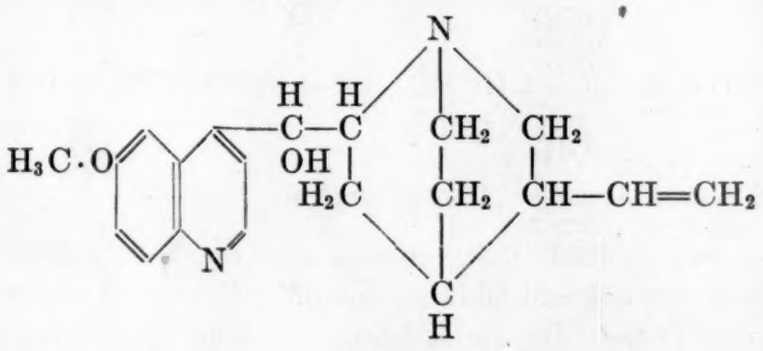


Pyridoxine

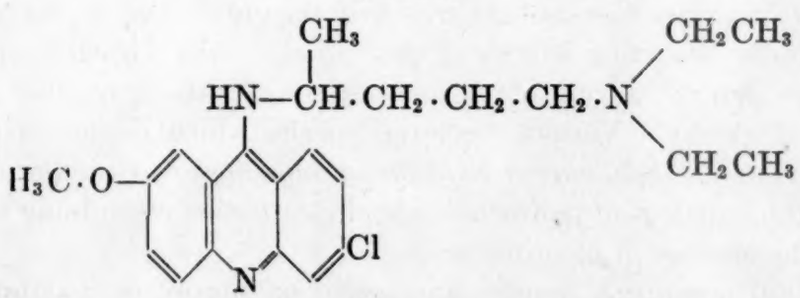


2,4-dimethyl-cyclotelluropentane-3,5-dione

It is unfortunate that the authors were unable to test experimentally their hypothesis, which appears reasonable, that the tellurium-containing compounds interfere with the metabolism of pyridoxine.



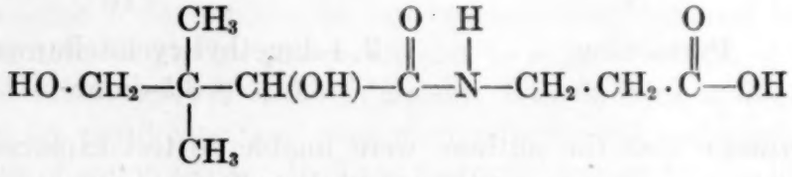
Quinine



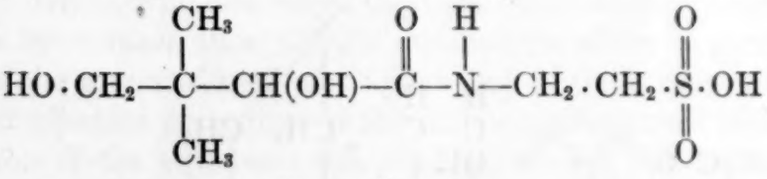
Atabrine

A most striking observation made recently by Seeler (44) suggests a relation between the action of quinine or atabrine and that of pyridoxine. It was shown that the therapeutic effect of these antimalarials, given by mouth to ducks infected with *Plasmodium lophurae*, is antagonized markedly when massive doses of pyridoxine are administered orally or subcutaneously. Seeler suggests that the vitamin, or a compound of similar structure, is essential for the life of the parasite and that quinine and atabrine act by competing with a pyridoxine-like compound for a position in some enzyme system of the parasite. As Seeler points out, a note of caution concerning this interpretation is offered by the results of Silverman and Evans (45), who found that on *E. coli*, *in vitro*, pyridoxine, under their experimental conditions, failed to affect the action of atabrine. Whether the results prove to be due to an inhibition of a truly competitive nature, or are less directly obtained, they are almost certainly of importance and could lead to improvements over drugs now available for the treatment of malaria.

*Pantothenic acid analogs.* Extensive studies have been carried out with various pantothenic acid-like compounds, particularly with the sulfonic acid analog, variously termed pantoyltaurine and thiopanic acid, and with the corresponding amide, pantoyltauramide.



Pantothenic acid



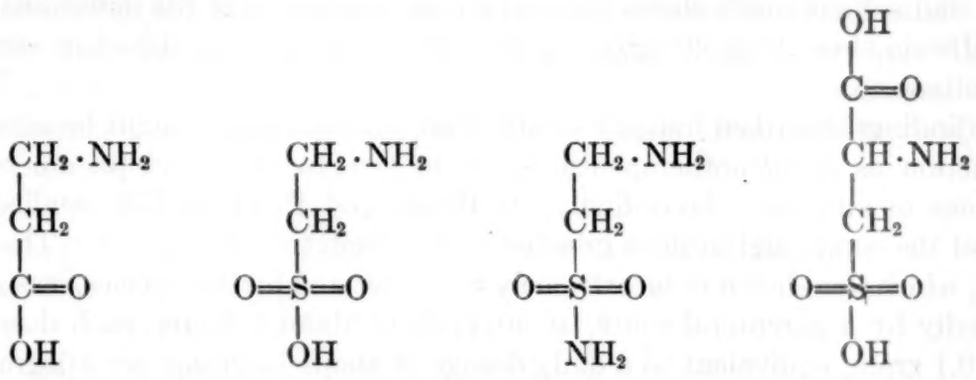
Pantoyltaurine

Snell (46) showed in 1941 that pantoyltaurine markedly inhibits the growth of *Lactobacillus arabinosus* and, in large amounts, the growth of a strain of yeast; this inhibition is reversed by the addition of calcium pantothenate, so that a truly competitive inhibition appears to occur. Essentially all the activity of racemic pantoyltaurine was shown to reside in the optical isomer that corresponds to the naturally occurring isomer of pantothenic acid. Snell found that the sensitivity to pantoyltaurine of various organisms which require pantothenic acid varies markedly. Various bacterial species which do not require pantothenic acid (*E. coli*, *Staph. aureus*, *Shigella paradysenteriae*, *Brucella abortus*) were not inhibited by analogs of pantothenic acid, a situation resembling that encountered with the analogs of nicotinic acid.

Since certain organisms require an exogenous supply of $\beta$-alanine, but not of the pantoic acid moiety of pantothenic acid, it is of interest to note that the

growth of *Saccharomyces cerevisiae* induced by β-alanine is *not* antagonized by pantoyltaurine. This observation might be interpreted as meaning that β-alanine in this case served otherwise than as a precursor of pantothenic acid; however, it appears more reasonable to suggest that pantoyltaurine fails to inhibit the growth of this strain of yeast for the same reasons it fails in other cases where pantothenic acid is synthesized by an organism.

Taurine, the sulfonic analog of β-alanine, was found by Snell (47) to be without influence on the utilization of the amino acid, and McIlwain (32) showed that taurine, taurine amide and cysteic acid were not inhibitory to any of the various organisms studied.




Hartelius (48) observed, however, that although β-aminobutyric acid does not influence the effect of pantothenic acid on the respiration of yeast, it does inhibit the activity of β-alanine.

The first of McIlwain's several studies of pantothenic acid derivatives appeared in 1942 (49). Eight compounds were examined, but of these only pantoyltaurine and its amide proved to be markedly inhibitory to bacterial growth. On a strain of *Streptococcus hemolyticus*, in the presence of $10^{-7}M$ pantothenate, pantoyltaurine and its amide (and to a lesser extent, homopantoyltaurine) markedly inhibited growth, an inhibition which was reversed by the addition of extra pantothenate.

In this and in other studies (50, 51), McIlwain has recommended the use of the convenient term, *antibacterial index*, which represents the minimal value of $C_I/C_M$, that is, the ratio of the concentration of inhibitor ($C_I$) just sufficient to prevent the growth of an organism, to the concentration of metabolite ($C_M$) present. Obviously, the smaller the antibacterial index the more effective is the compound as an inhibitor. Under the conditions described, and using *Streptococcus hemolyticus*, the following antibacterial indices were found: homopantoyltaurine—20,000; pantoyltauramide—2,000; pantoyltaurine—500. For pneumococci (types I, II or III) the antibacterial indices were: pantoyltauramide—10,000; pantoyltaurine—1,000. The pantoyltaurine used was racemic, so that the true activity is approximately twice that indicated in the above figures.

Bacteria which synthesize their own supply of pantothenate, such as *E. coli* and *Proteus vulgaris*, are not inhibited by these analogs of pantothenic acid. This failure cannot be attributed, however, to the synthesis of unduly large

amounts of pantothenate by the microorganisms; the mechanism of the resistance is at present unknown. One can only hypothesize that the synthesis may occur at such a locus within the cell that a sufficient concentration of the analog cannot be attained at the site of utilization, or that the metabolite synthesized is so efficiently used at the site of synthesis, or is used in such a modified form, that the analog cannot compete in the system involved. An even more difficult situation to explain is found in the case of *Proteus morganii*, an organism which requires exogenous supplies of pantothenate, but which fails to be inhibited by an excess of pantoyltaurine.

Calculation of the antibacterial indices of pantoyltaurine, pyridine-3-sulfonamide and sulfanilamide shows that, under the conditions of the determinations of McIlwain, hemolytic streptococci are most effectively inhibited *in vitro* by pantoyltaurine.

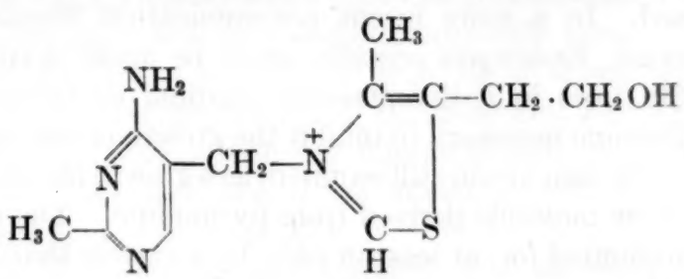
The findings described indicate clearly that pantoyltaurine might be expected to function as a chemotherapeutic agent in streptococcal and pneumococcal infections of animals. Accordingly, McIlwain and Hawking (52) studied the effect of the compound in mice infected with hemolytic streptococci. The substance, which was shown to be extremely well-tolerated by this species, was given repeatedly by a parenteral route, at intervals of about 8 hours; each dose was about 0.1 gram, equivalent to a daily dosage of about 12 grams per kilogram of body weight. The failure of the compound to protect the mice against streptococcal infection was accounted for by the very high levels of pantothenate in the blood of this species. Thus, when the experiment was repeated in rats, in which the level of pantothenic acid in the blood is lower than in mice, a high degree of protection was observed. During each day about 10 grams per kilogram were given subcutaneously, at intervals of about six hours, for a period of four days. The effective chemotherapeutic action exerted by the analog, under these circumstances, was abolished by the simultaneous administration of one-fiftieth the amount of pantothenate. The analog thus appears to interfere competitively with the utilization of pantothenic acid by the bacteria invading the tissues of the animals.

It is evident from the size and frequency of the dosage required to protect the infected rats that pantoyltaurine is without utility as a practical chemotherapeutic agent for the treatment of streptococcal infections. This failure may be attributed to the high concentration of the vitamin in mammalian tissues and to the extreme difficulty of maintaining an adequate concentration of the drug in the blood and other tissues. It is not inconceivable, however, that other modifications of the structure of pantothenic acid may be devised which would enable such compounds to compete effectively with pantothenic acid, and, at the same time to exhibit a more favorable rate of renal elimination, since that would appear to be a prominent cause of difficulty, and perhaps to affect organisms other than those dependent upon their environment for a supply of pantothenic acid. A compound with such characteristics might function as an effective chemotherapeutic agent, in addition to the sulfonamides and penicillin, for the treatment of streptococcal, pneumococcal, and other infections.

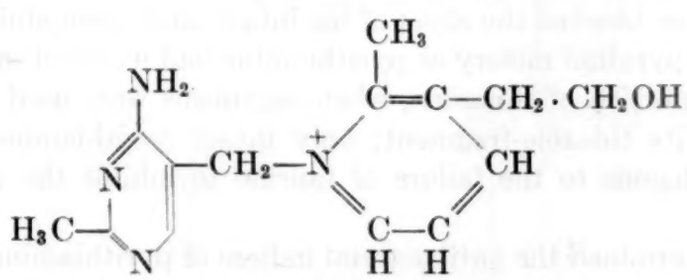
Barnett has described (53) the preparation of a group of substances closely related to pantoyltaurine, but none of these analogs is more active than pantoyltaurine as an inhibitor of *Lactobacillus arabinosus, in vitro,* or of *Streptococcus hemolyticus, in vivo.*

The levels of pantothenic acid and of its analog which were encountered by McIlwain in the blood of mice, strongly indicate that antagonism of the utilization of pantothenic acid in this species (or in the rat) would require fantastically large amounts of pantoyltaurine. Although a preliminary study conducted cooperatively by groups in California and Texas (54) indicated that feeding relatively large amounts of the analog to mice produced signs of pantothenic acid deficiency, this is now known almost certainly to have been artefactual (55, 56).

*Thiamine analog.* One of the most interesting substances which has been studied in connection with the blocking of essential metabolites is pyrithiamine, a compound in which the substituted thiazole ring of thiamine is replaced with a



Thiamine



Pyrithiamine

similarly substituted pyridine ring. It might be anticipated that this compound would have physicochemical properties closely similar to those of thiamine, since the work of Erlenmeyer (57) had indicated the close similarity of ring systems in which an —S— is replaced by —CH=CH—, or *vice versa.* Robbins (58) found that the growth of certain fungi which are dependent upon exogenous supplies of thiamine is markedly inhibited by the addition of this compound to the culture media.

Woolley and White have investigated the effect of pyrithiamine on mice (59) and as an inhibitor of the growth of various species of bacteria, yeasts and fungi (60, 61). Observations with microorganisms resembled those made with the analogs of nicotinic acid and of pantothenic acid, in that inhibition was found to occur only with those organisms which require for their growth an exogenous supply of the analogous vitamin. Organisms which synthesize thiamine are not

inhibited, even by large amounts of pyrithiamine, and the mechanism of resistance cannot be attributed to the synthesis of increased amounts of the corresponding vitamin. It would be interesting to determine, however, whether any pyrithiamine-sensitive organism could be "trained" to produce protective amounts of thiamine by culturing such strains in media containing progressively larger amounts of the analog. If such were the case the situation would be analogous to that which obtains in certain bacterial species that produce progressively larger amounts of p-aminobenzoic acid with continued exposure to progressively higher concentrations of a sulfonamide (62, 63, 64) On the other hand, under such circumstances, some bacterial species develop resistance to sulfonamides without any demonstrable increase in their production of paba (65). Woolley and White (60) showed that the resistance of bacterial species *initially* insensitive to pyrithiamine is not attributable to the production of large amounts of the vitamin, and no evidence of the production of any other inhibitor could be obtained. In a more recent communication Woolley (61) demonstrated that a yeast, *Endomyces vernalis*, could be made pyrithiamine-fast by transferring it 30 times in a thiamine-free medium containing 25 times the amount of pyrithiamine necessary to inhibit the growth of the parent strain one-half maximally. The new strain still required, as a growth factor, either thiamine or that portion of its molecule derived from pyrimidine. The resistance of the new strain was accounted for, at least in part, by a system that arose which was able to split the thiamine-analog into its pyrimidine and pyridine portions; the former apparently served the organism as a precursor for the synthesis of thiamine, which then blocked the effect of the intact analog remaining. It is worth noting that the pyridine moiety of pyrithiamine had no effect on the utilization of the thiazole portion of thiamine, when organisms were used that synthesize thiamine from its thiazole-fragment; only intact pyrithiamine was effective, a situation analogous to the failure of taurine to inhibit the utilization of $\beta$-alanine.

Wyss (66) determined the antibacterial indices of pyrithiamine with staphylococci and with *E. coli*. For the former, the index was about 700; for the latter, about 20,000. These results differ appreciably from those of Woolley and White (60), who with their strain of *E. coli*, obtained an index of more than 2,000,000, and an index of about 2,000 with *Staphylococcus aureus*.

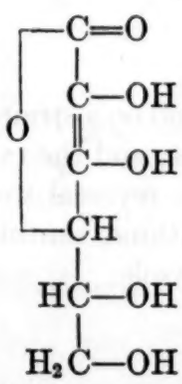
Woolley and White (59) have also studied the effect of pyrithiamine administration on weanling mice fed a highly purified diet. In this species the removal of thiamine from the diet causes only a gradual development of a deficiency syndrome, characterized by a progressive loss of appetite and loss of weight, with eventual death. However, with the exception of anorexia, the administration of adequate amounts of pyrithiamine caused the development of signs attributable to a severe thiamine deficiency. Within a period of 5 to 12 days, if sufficient pyrithiamine were given, and depending upon the ratio of it to the thiamine ingested, Woolley and White noted progressively: inactivity, tremors, convulsions, spasticity, extreme weakness, and finally death within about 3 days from the first appearance of the signs of thiamine deficiency. It was calculated

that about 40 molecules of pyrithiamine would nullify the effect of 1 molecule of thiamine. Some of the data showing cumulative effects indicate, however, that the ratio is even more favorable to antagonism, apparently not exceeding 10 to 1. It is obvious that pyrithiamine is an extremely active antagonist of thiamine.

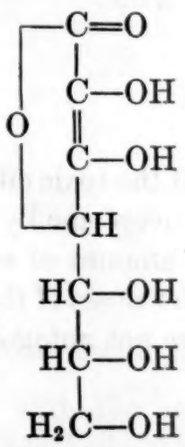
Unlike pantoyltaurine, pyrithiamine is apparently extremely slowly eliminated by the mouse, since delayed results were readily produced. For example, mice given 1.2 mgm. daily for 3 days, and then continued, without pyrithiamine, on a daily dosage of 2 micrograms of thiamine, grew normally for 6 days, but on the seventh day began to lose weight precipitously and to show the characteristic signs previously described. A single dose of 0.5 mgm. of thiamine on the ninth day produced an apparent cure within 24 hours. Cumulative effects were demonstrated by administering very small daily doses (20 micrograms of pyrithiamine with 2 micrograms of thiamine): after two and a half weeks without evidence of thiamine deficiency, the characteristic changes developed.

Were it not for the extremely toxic effect of pyrithiamine on the animal it might well have proved useful as a chemotherapeutic agent, so marked is its effect on bacteria dependent on external supplies of thiamine. However, all data indicate that the vital importance of thiamine and the minute amounts of the vitamin needed by both bacteria and animals completely prevent the utilization of the analog in the chemotherapy of infectious disease. As a nutritional tool it should continue to prove valuable.

*Ascorbic acid analog.* Particularly interesting is the work of Woolley and Krampitz (67) with glucoascorbic acid. Although at present it cannot be stated positively that the effect of this compound is attributable to metabolite-antago-




Ascorbic acid

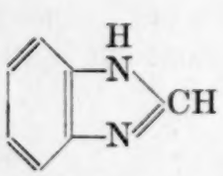



Glucoascorbic acid

nism, such an explanation would appear logical. When this compound was fed to mice at a 10 per cent level in a highly purified diet, there developed a syndrome characterized by failure in growth, diarrhea, subdermal hemorrhages, and death within 2 weeks. At a dietary level of 5 per cent the picture was similar, but death was either delayed or recovery occurred in spite of the continued ingestion of the compound. Such recovery was interpreted as signifying increased synthesis of the metabolite, with the function of which glucoascorbic acid interfered. In those animals which survived more than 2 weeks there developed an alopecia

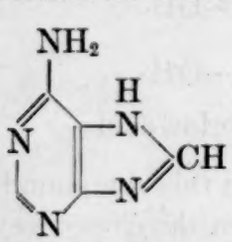
of the head and back that affected only those areas which did not become hairless as the result of a deficiency of inositol or of pantothenic acid. Two other interesting features were seen concerning the syndrome precipitated by this interesting compound: one, in mice it was not due to a simple antagonism of the utilization of ascorbic acid, since simultaneous feeding or injection of the vitamin did not prevent the development of the condition; two, the syndrome was prevented by the inclusion in the diet of various plant materials (powdered grass juice was effective at a minimal level of 10 per cent). The workers suggest that "certain plant substances contain a compound related to ascorbic acid in the normal course of its utilization, and that this substance is effective in overcoming the block to ascorbic acid metabolism caused by glucoascorbic acid." It would appear that through the use of glucoascorbic acid Woolley and Krampitz have discovered a clue to a new substance which is related to ascorbic acid and probably is a product of its metabolism. The close relationship of this new metabolite to vitamin C is also shown by the subsequent demonstration (68) in the guinea pig, a species dependent upon an exogenous supply of the preformed vitamin, that the deficiency syndrome characteristic of glucoascorbic acid, is completely prevented by the simultaneous administration of ascorbic acid.

*Analogs of purines and pyrimidines.* In surveying various compounds which might lend themselves to the synthesis of possible blockers of biotin metabolism, Woolley (69) examined the effect of benzimidazole on the growth of various organisms.

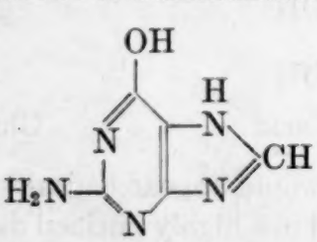


Benzimidazole

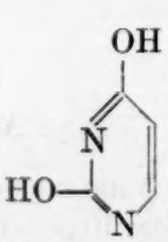
It was found that the toxic effect of this compound on a strain of *Saccharomyces cerevisiae* could be overcome by adenine or guanine, and the ratio of the amount of inhibitor to the amount of adenine required for reversal was about the same with high as with low doses of the drug. Hypoxanthine, xanthine, yeast adenylic acid and uracil were not antagonistic to benzimidazole.



Adenine



Guanine



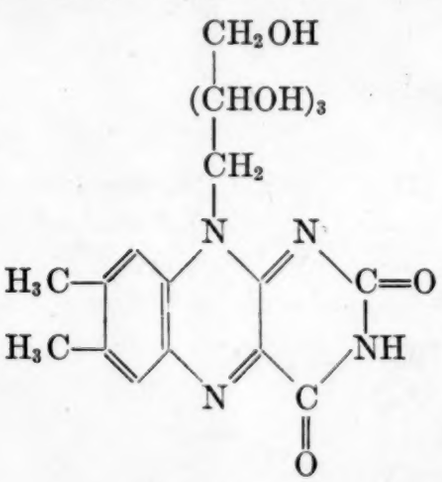
Uracil

Curiously, a compound even more closely related to adenine, namely, 4-aminobenzimidazole, was no more active than the unsubstituted compound. A remarkable observation, however, was that uracil, although inactive in reversing the effect of benzimidazole on the yeast, was the most active of all the compounds

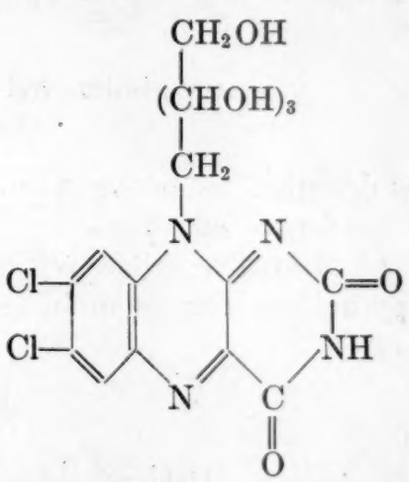
in causing an incomplete reversal of the effect of the inhibitor on *Streptococcus lactis R.* Also of interest was the finding that in mice the anesthetic effect of benzimidazole (10 mgm. intraperitoneally) was only very slightly modified by the administration of adenine sulfate (15 or 30 mgm.).

Presumably the effects described by Woolley (69) may be attributed to a competitive antagonism between benzimidazole and the naturally occurring compounds capable of reversing the growth-inhibiting effects of the substance. Although a relationship between the structures of the purines, adenine and guanine, and that of benzimidazole can be visualized by exchanging the pyrimidine ring for the benzene ring, and *vice versa*, it is more difficult to interpret the effect of the simple pyrimidine, uracil. In this connection Snell (70) has suggested that the growth factor-effect of uracil may not have been entirely separated from the inhibitor-reversal effect, since uracil is very effective in increasing the rate and extent of growth of the organism involved, and this substance was not included in the medium. Conceivably, if uracil were present in excess, adenine might have blocked the effect of imidazole on this bacterial species, as it did for yeast. Further study in this field may prove profitable.

*Riboflavin analogs.* A preliminary report by Kuhn and his co-workers (71) describes the effect of a compound in which the two methyl groups of riboflavin are replaced by chlorine atoms. The dichloro compound not only lacks growth-




Riboflavin



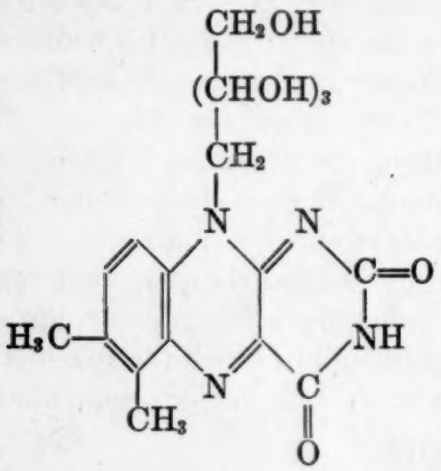

6,7-dichloro-9-ribityl-isoalloxazine

promoting properties, but effectively interferes with the action of riboflavin, so that the growth of certain bacterial species is blocked. Depending upon the duration of the experiment, the ratio of the concentration of inhibitor to that of metabolite required to permit 50 per cent of normal growth varied from 50:1 to 280:1 with *Staphylococcus aureus*, and from 25:1 to 165:1 for *Streptobacterium plantarum.* It is conceivable that this very active compound will prove useful, possibly as a chemotherapeutic agent, but almost certainly as a tool for the study of the metabolism of riboflavin.

Although the data available concerning this compound are very limited it would appear that its inhibitory effect possibly is *not* limited to those organisms unable to synthesize riboflavin. If this finding be confirmed, the substance may

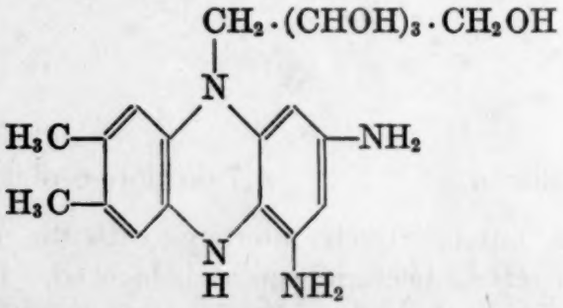
be added to those few compounds or groups of compounds which appear to be effective inhibitors, regardless of the ability of the organism to synthesize the respective metabolite. This group includes, in addition to the dichloro analog of riboflavin, benzimidazole, indoleacrylic acid, and of course, *par excellence*, the antagonists of p-aminobenzoic acid.

Emerson and Tishler (72) have described studies with isoriboflavin, an analog of the vitamin in which a methyl group is moved from the 7-position to the 5-position on the benzene ring. In rats a growth depression is produced by this compound, which is counteracted by an adequate amount of riboflavin. Woolley



5,6-dimethyl-9-ribityl-isoalloxazine
(Isoriboflavin)

also has described an analog of riboflavin (73), in which the phenazine ring is substituted for the alloxazine ring of the vitamin, a modification which may be considered to involve primarily the replacement, in alloxazine, of the 6-membered pyrimidine ring, containing two N-atoms, with a benzene ring. In

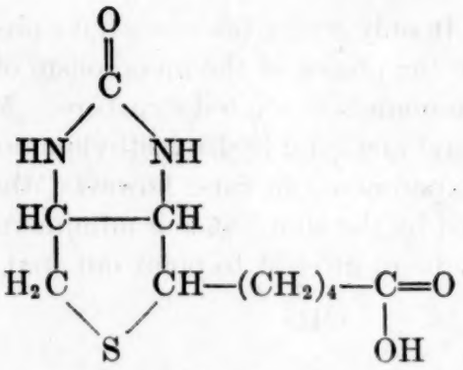


2,4-diamino-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine

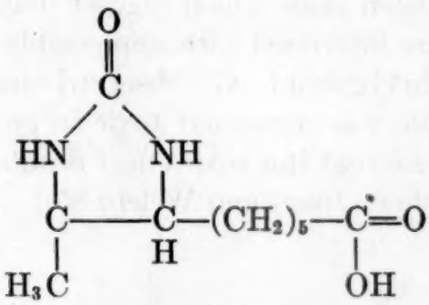
Woolley's compound amino groups also replace the oxygen atoms of alloxazine. This compound causes typical riboflavin deficiency in mice, a condition which is prevented by adding an excess of the vitamin to the diet.

Interesting results with biotin and its desthio-derivative have been reported by duVigneaud and his associates (74, 75) and by Lilly and Leonian (76).

Both groups find that desthiobiotin is for some organisms a growth-factor, while for others it is an antagonist of biotin. It is suggested by the Cornell



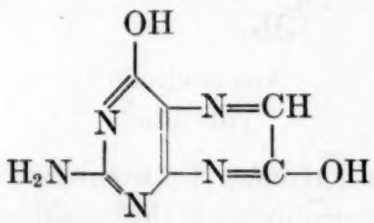
Biotin



Desthiobiotin

group, from their data with *Saccharomyces cerevisiae* in an actively growing state, that, for those organisms for which desthiobiotin is a growth factor, the substance is actually converted into biotin. For *L. casei*, however, and for a few other organisms unable to utilize desthiobiotin, the compound appears to inhibit the action of biotin competitively.

Another example of possible metabolite-antagonism is to be found in the investigations of Wright and Skeggs (77), who observed that the production of "folic acid" (*L. casei* factor) by *Aerobacter aerogenes* is significantly reduced by growing the organism in the presence of synthetic xanthopterin. However, the growth of this microorganism, and its production of biotin, are not affected by the pterin.

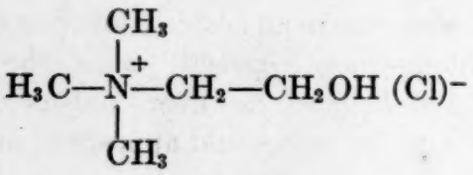


Xanthopterin

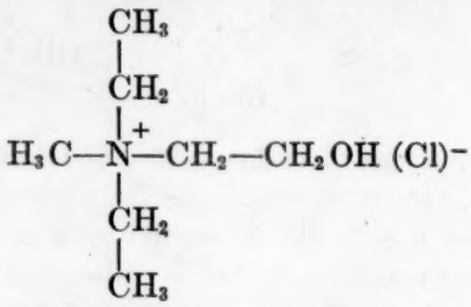
Further evidence of a possible relationship between "folic acid" and xanthopterin is afforded by the observations of Wright, Skeggs and Welch (78) showing that incubation of the hepatic tissue of rats with xanthopterin causes a significant increase in the yield of "folic acid." The evidence presently available indicates that xanthopterin interferes with the inactivation of "folic acid," a reaction presumably catalyzed by an enzyme system (79). Confirmation and explanation of the remarkable observations of Leuchtenberger, et al., on the inhibition of the growth of transplanted tumor tissue in mice by a crystalline "folic acid" (80) or by xanthopterin (81) is not yet available; if this effect of "folic acid" is verified, it is conceivable that the action of xanthopterin may be related to an interference with the destruction of "folic acid" absorbed from the alimentary tract of the animals.

A very large group of choline derivatives has been investigated for activity in preventing the development of fatty livers (82, 83) or of a hemorrhagic disease of the kidneys in mice or rats (84); various choline derivatives have also been examined for activity as avian growth-promoting agents and as agents exerting anti-perotic activity in chicks (85), and for their ability to substitute for choline

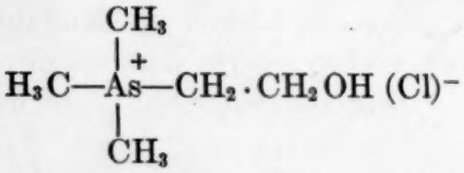
as a growth factor for pneumococci (86). In only a very few cases have observations been made which suggest that any of the phases of the metabolism of choline are interfered with appreciably by compounds of related structure. Moyer and duVigneaud (87) observed that diethyl-methyl-β-hydroxyethylammonium chloride was somewhat toxic in growth experiments in rats; however, there is evidence that this toxic effect is antagonized by the simultaneous administration of choline (Jukes and Welch, 85). It may be of interest to point out that even



Choline chloride



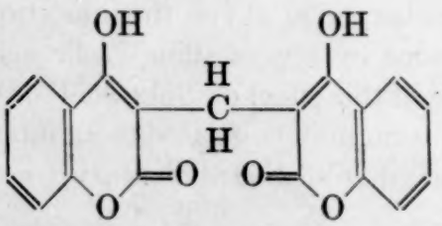
Diethyl-methyl-β-hydroxyethyl-ammonium chloride



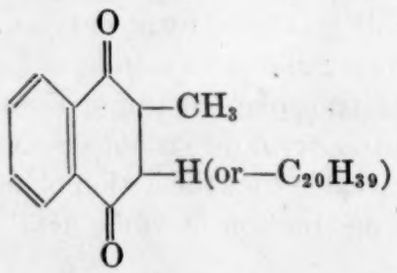
Arsenocholine chloride

the arsenic analog of choline, trimethyl-β-hydroxyethylarsonium chloride, does not appear to interfere appreciably with the transmethylating action of choline (88), and actually substitutes for choline in the synthesis of phospholipid (Welch and Landau, 89).

*Antagonism of vitamin K.* It has only recently been appreciated that the action of the coumarin derivative, isolated from spoiled sweet clover by Link and his co-workers (90), and now used in medicine to prolong the prothrombin time of blood, is due to its interference with the function of vitamin K. In



3,3′-methylene bis(4-hydroxycoumarin)

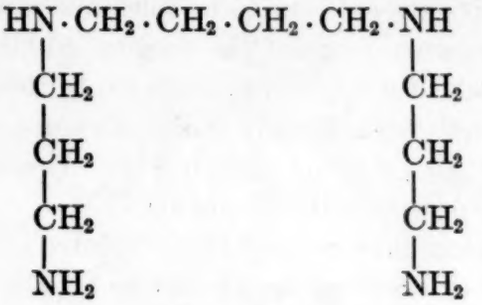


Vitamin K

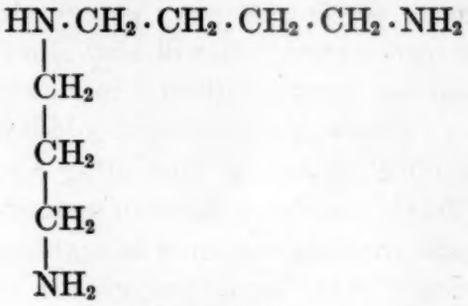
retrospect the structural resemblance between the compounds can be appreciated, and the actual relation is shown by the fact that the effects produced by the coumarin are removed by administration of relatively large amounts of vitamin K.

*Antagonism by polyamines.* Since the first report by Silverman and Evans

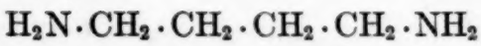
(45), several workers have studied the effect of various polyamines on the bacteriostatic influence of atabrine or propamidine (Snell, 91; Miller, Peters and Bosshardt, 92). There is no doubt that the action of atabrine or of propamidine on *E. coli*, and on some other organisms, is markedly reduced in the presence of tetraethylenepentamine, triethylenetetramine, spermine or spermidine, while the chemically related compound, putrescine, is inactive. The effects of these



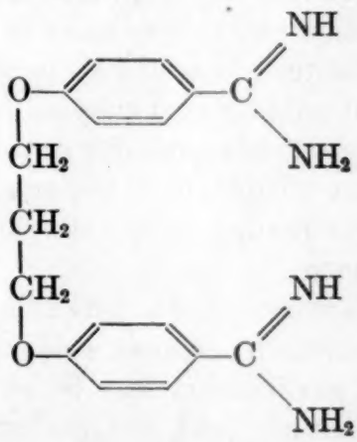
Spermine



Spermidine



Putrescine



Propamidine

polyamines are exhibited best within a certain range of concentration and in certain media. Whether they can be accounted for on the basis of competitive antagonism is not yet altogether clear. It is not inconceivable, however, that the compounds are related in structure to some substance thus far unidentified, the action of which is interfered with by atabrine and propamidine.

Discussion. The number of examples of metabolite-antagonism is now so great, the nature and function of the various metabolites so diverse, and the ways of modifying their structure to form antagonists so numerous, that the principle of interference with biological processes through the use of analogs of essential metabolites must now be considered as established. In some cases the interference can be explained most simply on the basis of a direct competition between the metabolite and its analog for some cellular component for which they both have great affinity; in other cases the data indicate that, in addition to competition, other factors operate. In the antagonism of p-aminobenzoic acid, and apparently in a few less well-defined cases, antagonism by related structures extends to metabolites normally synthesized by the organism whose function is affected. However, the majority of the interferences so far investigated

involve those forms of life unable to synthesize the essential metabolite the function of which is disturbed.

An explanation of the too common failure of analogs to block the utilization of endogenously formed metabolites is badly needed. Partly because of this failure the newer concepts have not led as yet to the development of a single compound of practical importance. There is still reason to believe, however, that this approach to the discovery of new drugs is rational, and principles are rapidly being formulated that will lead almost certainly to great therapeutic advances. It would be curious indeed if man, as a result of the groping which led to the discovery of the sulfonamides, should have found first the only group of compounds of practical utility in the antagonism of an essential metabolite. Assuming that that is not the case, let us examine the prospects for the future.

There are many avenues of approach which have not yet been explored. For practically every important growth factor or essential metabolite so far studied only a few analogous compounds have been prepared, among those which the mind can conceive and which the chemist may find a way to synthesize. A great many more derivatives must be made before rules relating chemical structure to antagonistic action can be formulated with any degree of precision. Actually it is not unlikely that rules in most cases will largely be restricted in application to one essential metabolite or to a group of metabolites having certain features in common. However, a few concepts of general significance concerning the relation of structure to the development of antagonistic properties appear to be developing.

The conversion of a carboxyl group to a sulfonic acid group or to a substituted or unsubstituted sulfonic acid amide has been particularly profitable. Examples of this modification may be seen, of course, in the analogs of p-aminobenzoic acid, nicotinic acid and pantothenic acid. The carboxyl group may also be changed to the —CO—R group, as in p-aminoacetophenone and in β-acetylpyridine. Isosteric modifications in which a ring —CH=CH— is converted to —S—, or *vice versa*, appear to be promising from the results obtained with pyrithiamine and thienylalanine, although in the paba field this type of modification has not led to particularly active structures, except in the case of certain nitro derivatives (25).

Many other possibilities for chemical modification remain and with some of these beginnings have been made. For example, the shifting of ring-alkyl groups, or their replacement with halogen, as in a modification of riboflavin; the lengthening of a chain, as with ascorbic acid; the replacement of oxygen atoms with those of sulfur, as might be done in hydroxyl groups, carboxyl groups, or imide linkages, in general, and a change in the reverse direction in certain specific cases; the replacement of N with C, P, As, Sb, Se, Te, S or O; the replacement of C with N or O; the introduction of alkyl or other groups into rings and chains; and other modifications, apparent to the chemist, but too numerous to mention. The important point to appreciate is that blocking-agents can be produced in a variety of ways but as yet no predictions can safely be made with regard to the effect of any given modification of chemical structure on the activity of a metabolic-analog.

The information which is steadily being acquired is of the greatest significance to chemotherapy in particular and to biological sciences in general. Of specific significance should be structural modifications of those essential metabolites active in the smallest amounts. With such compounds we may hope that certain microorganisms will prove to be relatively more dependent upon the essential metabolite than is the infected host. It may be suggested, from this line of reasoning, that analogs of biotin or of "folic acid" may be of promise and there is reason to believe that extensive work in the field of biotin chemistry, with such a goal in view, is now in progress.

Another field, separate from, but related to metabolite-antagonism, is developing in pharmacology. This concerns the further structural modification of compounds with known involvement in, or with influence on, various physiological processes. It is readily apparent that the effective blocking of the action of many hormones and drugs might have great practical utility. It is not improbable that certain drugs exert all or a portion of their desirable or undesirable effects on bodily mechanisms through competitive antagonism. It will suffice to comment upon a report by Swan and White (93) concerning certain compounds related to acetylcholine that act in the opposite manner, and function, therefore, as mydriatics and cycloplegics. Also, Unna (94) has reported on the use of N-allyl morphine in allaying the respiratory depression and other manifestation of toxicity of morphine. Although it is thought that the effect of atropine cannot be explained simply on the basis of competition with acetylcholine released at the endings of cholinergic nerves, a competitive type of antagonism appears to exist between pilocarpine and atropine, a phenomenon clearly demonstrated by Cushny a generation ago.

All the findings described emphasize that within the body of an animal the innumerable problems offered by the processes of absorption, distribution, excretion, metabolic alteration and toxicity, make our search for effective antimetabolites, particularly chemotherapeutic agents, a much more complicated task than the discovery of compounds with inhibitory properties that are counteracted by the corresponding physiological substance. Despite these difficulties it is reasonable to hope that the concept of competitive inhibition will be responsible for many useful developments in various fields of significance to biology and medicine. Application of these new principles permits the prediction that somewhat more in the future than in the past, and somewhat more by design than by chance, may we anticipate the discovery of new tools for the investigation of cellular metabolism and the treatment of disease.[3]

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